

Evaluation of Probiotics as Feed Supplements for Ostrich Chicks

Nikita Greenhill

**Thesis presented for the degree of Master of Science
in the Department of Molecular and Cell Biology
University of Cape Town**

March 2010

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Associate Professor Shez Reid, for her academic support and technical expertise during my time in her laboratory.

Thanks to Associate Professor Val Abratt for her encouragement and lessons in maintenance of 'Anaerobia', and to Dr. Di James for her sequencing expertise.

To Professor Tertius Brand and Bennie Aucamp at the Institute of Animal Production for their assistance with the feeding trials.

To all of the members of the Anaerobe Unit, for their camaraderie and advice.

Many thanks to the NRF, UCT, Ernst and Ethel Eriksen Fund, Twamley Foundation, Harry Crossley Foundation and the KW Johnston Bequest for their financial support.

And to my family, for their unwavering support.

CONTENTS

ABSTRACT	2
ABBREVIATIONS	4
CHAPTER 1 Introduction.....	5
CHAPTER 2 Identification and characterisation of strains and screening for mucin adhesion genes	29
CHAPTER 3 Physiological analysis of probiotic feeding trials.....	57
CHAPTER 4 DGGE analysis of probiotic feeding trials.....	81
CHAPTER 5 General conclusions	113
LITERATURE CITED	119

ABSTRACT

Farming of ostriches (*Struthio camelus*) is practised in many countries throughout the world, including South Africa. Production by these farms is limited by the high mortality rate of ostrich chicks. Chicks which lack a well established microbiota are more susceptible to potentially fatal pathogenic infections. Therefore, the mortality rate may be decreased by establishing the correct gut microbiota by the use of ostrich specific probiotic strains.

Five selected strains were conclusively identified and their mucin adhesion abilities characterised. Strain P1.2 was identified as *Enterococcus faecalis* by physiological tests and sequencing of the 16S rRNA gene and 16S – 23S intergenic spacer (ITS) region. The identity of strain 5934.3.1 was confirmed to be *Lactobacillus oris* by sequencing of the ITS region. The adhesion of the strains to mucin agar was assessed using a standardised method, involving enumeration of bacteria adhered to the agar by dilution and plating. Strains *Lactobacillus brevis* 512.3.1 and *Lactobacillus oris* 5934.3.1 were found to have adhesion to mucin agar which is comparable to that of *Lactobacillus rhamnosus* GG, a probiotic strain which has previously been shown to adhere to mucin. Strains 512.3.1 and 5934.3.1 were screened for the presence of the *mapA* and *msa* genes, which have roles in adhesion to mucin, by PCR and Southern hybridisation. The presence of these genes was not detected.

Random amplification of polymorphic DNA (RAPD) was evaluated as a method for tracking the presence of the probiotic strains in faecal samples. It was determined that this method was not appropriate for analysis of a large scale feeding trial. The five selected strains were included in an *in vivo* probiotic feeding trial, where ostriches were treated with an encapsulated mixture of the five strains and/or the antibiotic tylosin. The ostrich chicks which did not receive antibiotic treatment experienced a significant decrease in survival at four weeks. An increase in the weight gain of ostrich chicks receiving probiotic treatment was observed, although the statistical significance of this could not be determined due to a lack of

biological replicates at this point. Faecal samples collected from the ostriches at different intervals were cultured on a variety of complete and selective media. Titres of *Clostridium* spp. decreased at four weeks, after which the mortality rate decreased considerably.

The feeding trial was analysed by means of denaturing gradient gel electrophoresis (DGGE). Analyses showed no difference in the microbiota of the chicks due to either probiotic or antibiotic treatment, but the diversity of the microbiota was shown to change significantly as the birds aged. Sequencing of selected DGGE bands indicated the presence of *Clostridium populeti*, *Clostridium symbiosum* and *Clostridium hathewayi* in the faeces of day old chicks, but these are lost by week 9.

This study has shown that the five selected strains may be appropriate for use as probiotics for ostriches, and may be included in future studies for their effects on weight gain in ostrich chicks. It has also confirmed that the microbiota of ostriches changes with age, as it does in other tested animals.

ABBREVIATIONS

°C	degrees celsius	min	minutes
136.2.2J	<i>Lactobacillus johnsonii</i> 136.2.2J	ml	millilitre
136.3.1	<i>Bifidobacterium pseudolongum</i> subsp. <i>globosum</i> 136.3.1	mM	millimolar
		mm	millimetre
512.3.1	<i>Lactobacillus brevis</i> 512.3.1	MRS	de Man Rogosa Sharpe
5934.3.1	<i>Lactobacillus oris</i> 5934.3.1	mw	molecular weight
BHI	brain heart infusion	N	probiotic treatment
BSA	bovine serum albumen	n	number of replicates
C	control treatment	ng	nanograms
cfu	colony forming units	NT	probiotic and tylosin treatment
CT	control and tylosin treatment	OD600	optical density at 600 nanometres
d	day	p	plasmid
DAPI	4', 6-diamidino-2-phenylindole	P1.2	<i>Enterococcus faecalis</i> P1.2
DGGE	denaturing gradient gel electrophoresis	PBS	phosphate buffered saline
DIG	digoxigenin	PCR	polymerase chain reaction
DNA	deoxyribonucleic acid	RAPD	random amplification of polymorphic DNA
dNTP	deoxyribonucleotide triphosphate	RFLP	restriction fragment length polymorphism
EDTA	ethylenediaminetetra acetic acid	rpm	revolutions per minute
EU	European Union	rRNA	ribosomal ribonucleic acid
FISH	fluorescent in situ hybridisation	s	seconds
g	gram	SDS	sodium dodecyl sulphate
gDNA	genomic deoxyribonucleic acid	SS	Salmonella Shigella
GIT	gastrointestinal tract	ssDNA	single stranded deoxyribonucleic acid
GRAS	generally regarded as safe	TAE	tris acetate EDTA buffer
h	hours	Tris	tri(hydroxymethyl)aminomethane
H'	Shannon Wiener Index	TSC	Tryptose Sulphite Cycloserine
HRM	high resolution melt	U	unit
ITS	16S-23S intergenic spacer region	UPGAMA	unweighted pair group method with arithmetic averages
kb	kilobases		
l	litre	UV	ultra violet
LB	Luria-Bertani broth	V.cm ⁻¹	Volts per centimetre
LGG	<i>Lactobacillus rhamnosus</i> GG	wk	week
log	logarithmic	w/v	weight per volume
M	molar	λDNA	lambda deoxyribonucleic acid
mg	milligram	μ	micro

CHAPTER 1

INTRODUCTION

1.1. Probiotics and Their Effects on Health.....	7
1.2. Diseases in Ostriches	9
1.3. Comparison of the Gastrointestinal Tracts of Ostriches and Poultry	10
1.4. Composition of Poultry Microbiota.....	12
1.5. Selection of Probiotics for Poultry	14
1.5.1. Intestinal Adhesion in Probiotic Bacteria	16
1.5.1.1. Binding to Intestinal Surfaces.....	16
1.5.1.2. Adhesion to Intestinal Mucus	17
1.5.1.3. Intestinal Mucins	17
1.5.1.4. Surface Proteins Implicated in Mucin Adhesion	19
1.5.1.4.1. LPXTG – Motif Proteins.....	20
1.5.1.4.2. Anchorless Housekeeping Proteins.....	22
1.5.1.4.3. Transporter Proteins	23
1.6. Delivery Mechanisms for Probiotics	23
1.7. Probiotics Used in Poultry	24
1.8. This Study.....	25

Ostrich farming has been an established practice in South Africa for 150 years. In more recent years an increase in demand for the meat and leather products has led to this farming type spreading to many countries, including Korea, U.S.A., Belgium, Italy, Australia and Russia (Huchzermeyer, 2002; Shivaprasad, 1993; Carbajo, 2006). There is a high mortality rate among ostrich chicks associated with pathogenic infection (Huchzermeyer, 2002; Shivaprasad, 1993). Chicks which lack a well established microbiota are more susceptible to infection, thus the mortality rate could potentially be decreased by ensuring the establishment of the correct gut microbiota. This presents an opportunity for a novel use of probiotics.

The diet of wild ostriches largely consists of green grasses, berries, seeds, succulent plants and small insects (Aganga *et al.*, 2003), while farmed ostriches are often fed lucerne, or an artificial feed blend. Green grasses are included in artificial feed to stimulate pecking. Ostrich feed can be pelleted, or in a meal form. Regular farming practice, to ensure ostrich health, uses three feed types, high protein starter feed, grower feed and maintenance feed (Cooper and Mahrose, 2004). These are commonly formulated from maize meal, fish meal, white alfalfa, carcass meal, and additional nutrients such as methionine, lysine, salt and various vitamins and minerals. Ostriches should also have access to small, round stones, which, after ingestion, aid the physical grinding of food in the gizzard (Aganga *et al.*, 2003).

From the moment of hatching, the once sterile alimentary canal of a bird is colonised by a host of bacteria from the environment. Wild birds may receive an initial inoculum from the egg shell during hatching, as it is covered in bacteria from the mother's intestine. This opportunity for the colonisation by microflora from a healthy adult may be missed where eggs are hatched artificially, due to sterile conditions used in hatcheries (Apajalahti and Kettunen, 2006). In addition to this, ostriches are known to be coprophagous, consuming faecal material from their parents, which provides an inoculum of microbiota (Aganga *et al.*, 2003). This is another source of colonising gut bacteria native to wild ostriches, which is

available to birds raised with their parents, as seen in Figure 1.1, but not to most intensively farmed ostrich chicks.

1.1.Probiotics and Their Effects on Health

Probiotics have been defined as “live microbial food supplements which beneficially affect the host animal by improving its intestinal microbial balance” (Gomes and Malcata, 1999). Probiotics are increasingly being used as a tool to improve the health of farmed animals. This strategy is employed due to the observed effects of the gastrointestinal microbiota of broiler chickens on intestinal infections and the immune response, both of which are important in the prevention of pathogenic infections in the gut (Lu *et al.*, 2003).

The effects of a probiotic on a host include modifying the microbiota, competitive exclusion, release of nutrients and regulation of the immune system. The primary effector of this beneficial effect is hypothesised to be competitive exclusion, whereby the colonisation of the gut by these commensals prevents the establishment of pathogenic strains (Gabriel *et al.*,



Figure 1.1: An adult ostrich with her chicks in a farm enclosure with lucerne plants. These chicks are able to practise coprophagy, ingesting faecal matter from their mother. (Western Cape Department of Agriculture, Institute for Animal Production, 2005).

2006; Netherwood *et al.*, 1999). While the precise mechanism of competitive exclusion remains unknown, several factors have been implicated; competition of a probiotic with a pathogen for nutrients, as well as receptors in the gut (Mead, 2000; O'Sullivan *et al.*, 2005). Production of inhibitory compounds, including volatile fatty acids, lactic acid, peroxide and free radicals can deter growth and colonisation by pathogens (Mead, 2000; Gomes and Malcata, 1999; Gabriel *et al.*, 2006; O'Sullivan *et al.*, 2005; Vahjen *et al.*, 2002). Probiotics may also promote the production of the immunologic and nonimmunologic defence barriers in the gastrointestinal tract, and modify the permeability of the mucosa (Gomes and Malcata, 1999). Pathogenic colonisation may be inhibited by the acidic or low redox potential of the gastrointestinal environment, created by the host microbiota (Gabriel *et al.*, 2006). Modification of the microbial composition can be accomplished by using all of these mechanisms (Patterson and Burkholder, 2003).

In poultry, the composition of the intestinal microbiota changes as the bird ages (Gabriel *et al.*, 2006). It has been found that a lack of intestinal microbiota in an animal has adverse effects on its health, and that these effects can be reversed by allowing this gnotobiotic animal to be colonised by the appropriate microbiota (O'Sullivan *et al.*, 2005).

Probiotics aid the host in a number of ways including the digestion of nutrients which the host cannot process and creating usable degradation products (Gabriel *et al.*, 2006; Netherwood *et al.*, 1999). *Lactobacillus reuteri* has the potential to improve the absorption of nutrients by increasing the intestinal villus height of chickens (Timmerman *et al.*, 2006).

Due to the role assigned to the microbiota in the development and establishment of the immune system, alteration of the microbial balance in the gut results in a change in the immune response (Timmerman *et al.*, 2006; Gabriel *et al.*, 2006; O'Sullivan *et al.*, 2005). These changes include stimulation of the innate immune response, via activation of phagocytosis and cytokine synthesis by macrophages, and attenuation of the inflammatory

response (Gabriel *et al.*, 2006). Work in human cell lines indicates that detection of bacterial molecules such as peptidoglycan and lipopolysaccharide can result in activation of both the innate and adaptive immune responses (Kelly and Conway, 2005). While the innate immune response of chickens has not been completely described, it is thought that innate mechanisms similar to those found in mammals function in recognition of bacterial molecules (Brisbin *et al.*, 2008). Results obtained by Haghighi *et al.* (2006) suggested that treating unimmunised chicks with probiotics induced the production of natural antibodies. Natural antibodies have broad specificity, and are produced prior to exposure to an antigen. There is an established relationship between the natural antibody and specific antibody response.

1.2.Diseases in Ostriches

There are many diseases affecting the health of young ostriches that together result in a mortality rate of 60 – 100 % (Shivaprasad, 1993). South African ostrich research has shown that 78 % of ostrich chicks die before 90 days with the vast majority (46 %) dying within 28 days of hatching. One of the risk factors for death during this period was a mass of less than 762.5 g one day after hatching (Cloete *et al.*, 2001). A study of ostriches submitted for necropsy in California, USA, showed that approximately 80 % of the birds which died were under the age of 84 days. Most of the ostriches from this study were found to have some disease of the gastrointestinal system (Shivaprasad, 1993). The major diseases contributing to the high mortality rate are enteritis and fading chick syndrome. Enteritis in young chicks is usually caused by Clostridia and Gram negative bacteria. The source of fading chick syndrome is unknown, but is associated with an absence of gastric contractions, making complete digestion of food impossible for the ostrich. The condition is observed as weight loss and cessation of growth in chicks (Huchzermeyer, 2002). Other noted diseases in ostrich chicks, such as colitis and candida infections of the proventriculus, are also related to gastrointestinal infections (Shivaprasad, 1993).

Poor gut colonisation by normal microbiota contributes to pathogenic infection (Huchzermeyer, 2002). This could be prevented by the administration of a suitable probiotic that allows the development of a healthy gastrointestinal tract. This, in turn, should prevent colonization of the gut by pathogens and stimulate the immune response. As the normal ostrich microbiota has yet to be fully characterised, the nearest available model is that of poultry.

1.3. Comparison of the Gastrointestinal Tracts of Ostriches and Poultry

The use of probiotics in poultry can be considered as a model for the development of probiotics for ostriches. However, there are significant differences between ostriches and poultry. Unlike poultry, ostriches have no crop. Instead, the oesophagus empties food directly into the proventriculus. In ostriches, the large intestine appears after the caeca, and is much longer than the small intestine, as seen in Figure 1.2. The entire hindgut is important for the digestion of the ostrich's fibrous food, which consists mainly of tough, drought resistant plants (Cooper and Mahrose, 2004). In contrast, the colon, or large intestine of poultry is relatively small (Figure 1.3). While the microbiota of chickens aids in the digestion of some carbohydrates in the caeca, it appears to be unable to digest fibrous substances, such as cellulose. It should be noted, however, that this is not true for all poultry. The microbiota of turkeys is able to digest cellulose (Gabriel *et al.*, 2006).

There is limited information available on the composition of the microbiota of ostriches (Matsui *et al.*, 2010a). While culture-based studies of the microbiota of ostriches have been completed, many of these were focused on fibrolytic bacteria. Sequencing of a 16S rRNA gene clone library constructed from the ceecal contents of ostriches showed the presence of many species of bacteria, including *Clostridium* spp., *Lachnospira* spp., *Ruminococcus* spp., *Bacteroides* spp., *Prevotella* spp. and a novel *Fibrobacter* sp. (Matsui *et al.*, 2010b). Interestingly, no members of the genus *Lactobacillus* were detected in this study. This is the

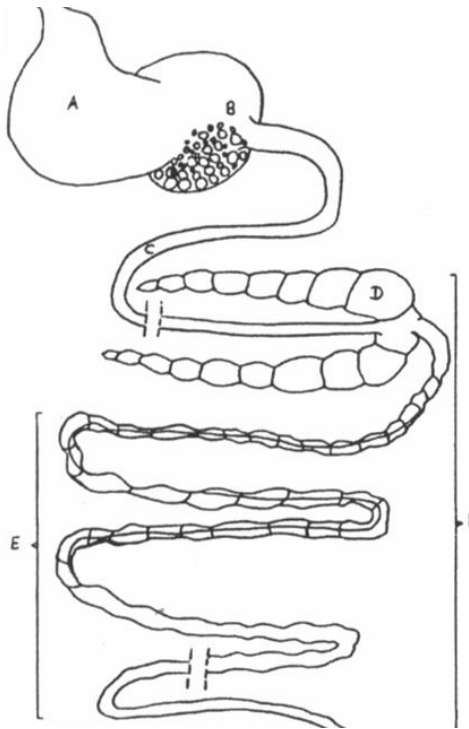


Figure 1.2: Diagram of the digestive tract of an ostrich (modified from Cooper and Mahrose, 2004). (A) proventriculus; (B) gizzard; (C) small intestine; (D) caeca; (E) colon or large intestine; (F) hindgut.

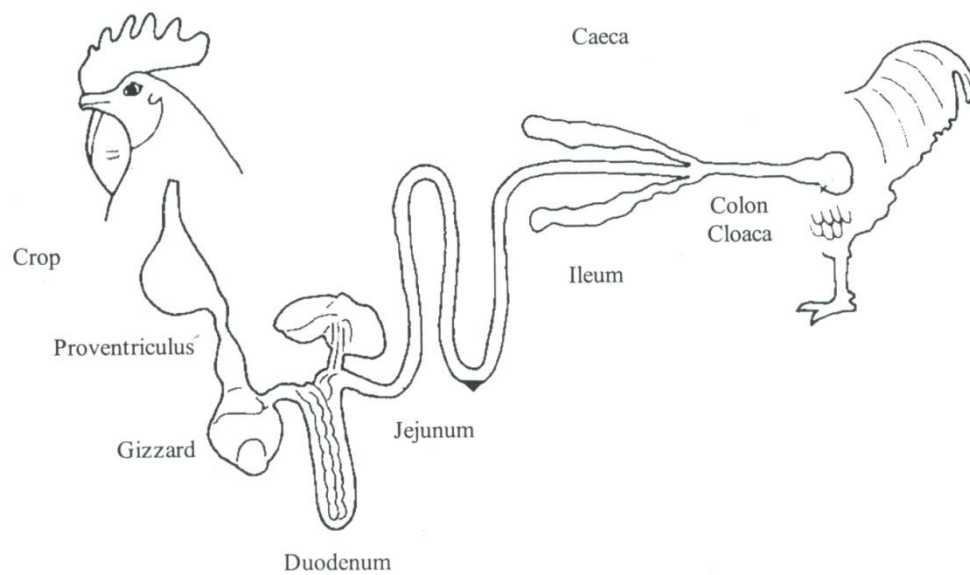


Figure 1.3: Diagram of the digestive tract of a chicken (modified from Gabriel *et al.*, 2006).

only large scale culture independent analysis of the caecal microbiota of ostriches that has been done to date, and there has been no reported research on probiotic strains specific to ostriches. The composition of poultry microbiota is discussed in section 1.4.

The physiological differences between poultry and ostriches are likely to be reflected in differing compositions of gut microbiota. Therefore, the value of poultry as a model system for probiotic treatment of ostriches lies in the similarity of the strategy to be used, rather than in the specific identity of probiotic strains.

1.4.Composition of Poultry Microbiota

Many studies have been done in order to characterise the composition of poultry microbiota. While there are a few genera, such as the lactobacilli and bifidobacteria, which are usually identified in culture-based studies, the organisms routinely identified are heavily dependent on the screening methods used (Haghighi *et al.*, 2006; Lu *et al.*, 2003). In order to ensure that the results obtained reflect the microbiota of poultry in their natural environment, it is essential that the poultry sampled have been raised on unsupplemented feed, i.e. without antibiotics or additional protein supplementation. During the production of supplemented feeds, contamination with microorganisms may occur, which may subsequently affect the composition of the gut microbiota (Lu *et al.*, 2003).

Gender and breed are some of the factors affecting the composition of an animal's microbiota. Microbial composition varies between individuals of the same breed and even within the individuals as they age (Gabriel *et al.*, 2006; Lu *et al.*, 2003). The composition of an individual's microbiota is dependent on several factors, including the bacteria present in the initial inoculum after hatching, the structure of the individual's intestinal epithelium, and the diet (Apajalahti and Kettunen, 2006). The use of culture dependent methods does bias the results of these studies, as it selects for culturable bacteria, and as a result, it has been

estimated that only one quarter of the microbiota of chickens has been characterised to date (Mead, 2000). Recently, the composition of the microbiota of poultry has been examined using molecular methods, such as amplification and sequencing of 16S rRNA genes in samples taken from the gut of healthy adult chickens (Lu *et al.*, 2003; Zhu *et al.*, 2002; Gong *et al.*, 2002). Molecular methods have supported the results obtained by culture based methods, and have allowed a greater understanding of the diversity of the gut microbiota (Lu *et al.*, 2003). In addition, the use of 16S rRNA gene sequencing has shown that some poultry microbiota is related to the putative gut microbiota of other genera, especially bovine and human microbiota (Gong *et al.*, 2002).

Most of the caecal microbiota of chickens consists of strict anaerobes, but some facultative anaerobes are also present. The ileum is colonised mostly by facultative anaerobes (Gabriel *et al.*, 2006).

A few studies performed 16S rRNA analysis of the caecal microbiota of chickens, but these studies were all completed using slightly different parameters, and different results were obtained (Lu *et al.*, 2003; Zhu *et al.*, 2002; Gong *et al.*, 2002; Gong *et al.*, 2007). While all detected large numbers of *Clostridium* spp. in their samples and two groups detected *Lactobacillus* spp. (Lu *et al.*, 2003; Gong *et al.*, 2007), Zhu *et al.* (2002) showed that *Sporomusa* spp. were present, and Gong *et al.* (2002; 2007) observed the presence of *Ruminococcus* spp. In addition to the *Clostridium* spp., Lu *et al.* (2003) found many *Fusobacterium* spp. and *Bacteroides* spp., and Gong *et al.* (2007) also found sequences related to *Faecalibacterium* spp., and *E. coli*. The difference in the results of these studies is probably due to a difference in the chickens' diets (Gabriel *et al.*, 2006), and the treatment with growth-promoting antibiotics (Gong *et al.*, 2007). Therefore the results of Gong *et al.* (2007) reflect the microbiota of poultry treated with zinc bacitracin.

Three of the studies discussed above examined the composition of the microbial community of the ileum, in addition to that of the caecum (Lu *et al.*, 2003; Zhu *et al.*, 2002; Gong *et al.*, 2007). All three studies determined *Lactobacillus* spp. to be the largest component of the ileal microbiota. *Enterococcus* spp. were detected by Lu *et al.* (2003) and Gong *et al.* (2002). Other predominant species found by Lu *et al.* (2003) included *Clostridiaceae* and *Streptococcus* spp.

A study of the microbiota of the duodenum and jejunum in chicks treated with zinc bacitracin showed that the predominant bacteria present were lactobacilli (Gong *et al.*, 2007).

1.5. Selection of Probiotics for Poultry

In vivo testing of potential probiotics is an expensive exercise, as replicate groups of animals are required in order to obtain data that can be subjected to statistical analysis. *In vitro* screening can be used to identify strains which are potentially good probiotics, reducing the number of strains which are subjected to *in vivo* testing. Such screening takes into account many factors which increase the chances of the bacterial strain colonising the gut and being beneficial to the host. Strains which are human or poultry pathogens are eliminated from further study. Ideal probiotics should have the following characteristics: originating from the host, tolerance to acidic conditions and bile salts, an ability to adhere to intestinal surfaces, such as epithelium or mucus, produce compounds which inhibit pathogens, modulate immune response, survive and colonise the GIT (gastrointestinal tract), and survive packaging and storage (Ehrmann *et al.*, 2002; Jacobsen *et al.*, 1999; Reid, 1999; Patterson and Burkholder, 2003). The production of antimicrobial compounds by the probiotic is a useful characteristic when the probiotic is to be used for the prevention of colonisation by a particular pathogen, e.g. *Salmonella* spp. (Reid, 1999; Gabriel *et al.*, 2006), as is the production of an enzyme that aids digestion by the host (Gabriel *et al.*, 2006; Netherwood *et al.*, 1999).

Once these primary requirements have been fulfilled, a selection of putative probiotic strains may be subjected to *in vivo* trials. These trials must show that the probiotic has a positive effect on the host, and that it has the ability to colonise the gut (O'Sullivan *et al.*, 2005). In addition to this, the viability of a probiotic strain should be considered. It is vital that a probiotic is viable at the moment it is consumed by the host (Mead, 2000). The effects of storage on the viability of the strain should be taken into account. If the candidate probiotic is for commercial use, it must be able to be cultured in large scale (Gomes and Malcata, 1999).

The methods used in the course of an *in vivo* trial are important, as limited sampling or methods of analysis can prevent detection of the viable probiotic strain, as well as detection of changes in the unculturable portion of the microbiota. Animals receiving different treatments should be physically separated in order to prevent horizontal transfer of bacterial strains, and control groups treated with sub-therapeutic doses of antibiotics should be included to compare the effects of the two treatments (Patterson and Burkholder, 2003). An appropriate number of samples should be taken to enable statistical analysis of experiments, and stored appropriately, as storage methods have been found to affect results of analyses of microbial communities (Wintzingerode *et al.*, 1997).

Culture studies are useful in determining large variations in the culturable microbiota due to treatment, and in detection of the viable probiotic. A number of selective media may be used to assess different groups of bacteria (Fujiwara *et al.*, 2001; De Angelis *et al.*, 2007). Strains may be selected from those cultured for further analysis using rapid amplification of polymorphic DNA (RAPD) or sequencing of the 16S rRNA genes (De Angelis *et al.*, 2007). A complementary culture-independent analysis should be carried out to find changes in the unculturable microbiota. A variety of methods could be used to achieve this, including sequencing of 16S rRNA genes found in faecal samples, quantitative PCR, fluorescent *in situ*

hybridisation (FISH) and denaturing gradient gel electrophoresis (DGGE) (Abbas Hilmi *et al.*, 2007; Rochet *et al.*, 2006; Tannock *et al.*, 2000).

It is important to remember that while survival of passage through the GIT by a bacterial strain has a link with some *in vitro* characteristics, these do not guarantee that the microorganisms will be able to persist and colonise the gut. Therefore, *in vitro* screening should always be followed by *in vivo* trials (Jacobsen *et al.*, 1999), and these trials should be assessed using both culture dependent and culture independent methods of analysis. This ensures that a full view of the uncultured microbiota is obtained, and the viability of the probiotic at the sampling site is confirmed (Patterson and Burkholder, 2003).

1.5.1. Intestinal Adhesion in Probiotic Bacteria

1.5.1.1. Binding to Intestinal Surfaces

The ability to bind to intestinal epithelium or mucus is one of the characteristics of the ideal probiotic (Patterson and Burkholder, 2003). Bacteria are routinely tested for the ability to bind to intestinal epithelium in human intestinal cell lines, such as Caco-2 cells (Tuomola and Salminen, 1998; Tallon *et al.*, 2007), and while there is an avian intestinal epithelium cell line (Velge *et al.*, 2002), there is no equivalent system available for ostriches. Alternatively, the adhesion of the strain to resected intestinal tissue samples, an *ex vivo* model, could be determined (Vesterlund *et al.*, 2005; Edelman *et al.*, 2002). Comparisons of the adhesion to an intestinal epithelial cell line, basement membrane proteins and mucin shows that the adhesion of a single strain depends on the experimental model employed (Tallon *et al.*, 2007). While these models do not show all of the complex interactions occurring in the gut, they do provide screening methods for bacteria which adhere to intestinal surfaces in general (Muñoz-Provencio *et al.*, 2009), and provide a method for screening for proteins involved in these interactions (Wang *et al.*, 2008).

1.5.1.2. Adhesion to Intestinal Mucus

Mucus lines the intestinal epithelium, providing the first surface to which intestinal bacteria have an opportunity to adhere (Kirjavainen *et al.*, 1998). The mucosal lining may prevent the infection of epithelial cells by pathogens, since bacteria must penetrate this lining before invading the epithelial cells (Donoghue *et al.*, 2006). Adhesion to mucus has been investigated *in vitro* by measuring binding of radioactively labelled bacterial strains to intestinal mucus extracted from faecal samples (Roos and Jonsson, 2002; Kirjavainen *et al.*, 1998; Ouwehand *et al.*, 1999; Rinkinen *et al.*, 2003). Allowing the adhesion of unlabelled bacteria prior to measurement of the adhesion as described above has been used to investigate the effects of other bacterial strains on the adhesion of probiotic strains. These experiments have shown that the normal microbiota does not influence the adhesion of probiotic strains (Ouwehand *et al.*, 1999) and that the presence of some probiotic strains can enhance the adhesion of other probiotic strains to intestinal mucus (Ouwehand *et al.*, 2000). Rinkinen *et al.* (2003) also used this technique with intestinal mucus from several different animals to show that the ability to adhere to intestinal mucin is reliant on the bacterial strain and not the host species. Kirjavainen *et al.* (1998) reported the adhesion properties of probiotic strain *Lactobacillus rhamnosus* GG, and this strain is used as a positive control for other binding studies (Ouwehand *et al.*, 1999; Ouwehand *et al.*, 2000). Mucin is continuously degraded by the action of bacteria (Kirjavainen *et al.*, 1998).

1.5.1.3. Intestinal Mucins

The surface of the intestinal epithelium is coated by a layer of intestinal mucus (Figure 1.4). The mucus layer is comprised of glycolipids and mucins, which are large, highly

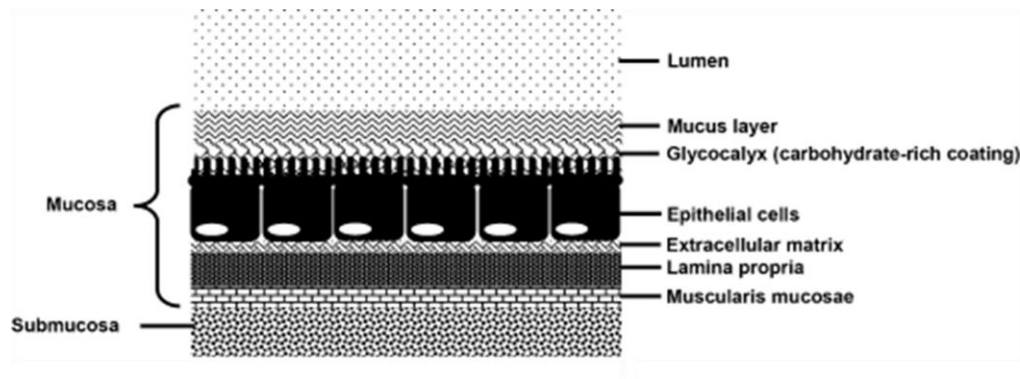


Figure 1.4: Diagram of the components of the human mucosa and submucosa. Modified from Vélez *et al.* (2007).

glycosylated proteins (Vélez *et al.*, 2007). Mucins have been defined as glycoproteins which consist of 50 % (by weight) or more O-linked oligosaccharides, most of which are linked to serine and threonine residues of mucin domains (Axelsson *et al.*, 1998; Lidell *et al.*, 2006).

Mucin monomers may interact and form disulphide bonds. The result of these bonds is a gel-like morphology (Lidell *et al.*, 2003).

Substantial amounts of research have been conducted on the human mucin genes; homologues of MUC-2, a gene encoding a polymeric mucin, occur in humans, chimpanzees, dogs, mice, and chickens (HomoloGene accession number 80094). Mucins function in protection of the intestinal epithelium from chemical damage, and in the binding of the intestinal microbiota (Carlstedt *et al.*, 1993; Axelsson *et al.*, 1998). In addition to this, damage to the mucin is associated with some pathogenic infections, and the binding of intestinal mucin by other bacteria may prevent adhesion and infection by some pathogens (Lidell *et al.*, 2006). Mucin may also be used by bacteria as a fermentation substrate (Donoghue *et al.*, 2006), and the presence of mucin in the growth medium of some *Lactobacillus reuteri* strains induces strong adhesion properties (Jonsson *et al.*, 2001).

Since adhesion to epithelial cells is only possible for cells which have already passed through the intestinal mucus lining, screening for adhesion to intestinal mucus and mucins may be the

most appropriate methods of initial screening (Van den Abbeele *et al.*, 2009). Adhesion to intestinal mucus has been investigated in order to gain insight into the interaction between bacteria and the intestinal surface. However, in order to find specific bacterial cell components responsible for such interactions, more defined models, such as epithelial cell lines and partially purified mucins, have been used (Wang *et al.*, 2008; Vélez *et al.*, 2007).

1.5.1.4. Surface Proteins Implicated in Mucin Adhesion

There are several individual surface factors which play a role in adhesion to the intestinal lining (Antikainen *et al.*, 2009). While such proteins are widely investigated for their role in adhesion to intestinal surfaces, non-proteinaceous compounds, such as lipoteichoic acids, have been shown to be involved in the adhesion of *Lactobacillus* to epithelial cells (Granato *et al.*, 1999). There are five types of surface proteins that have been implicated in adhesion, namely; surface layer proteins, LPXTG – motif protein anchorless housekeeping proteins, transporter proteins and representatives of protein families that are not usually associated with any of these categories (Antikainen *et al.*, 2009). Only the proteins which have been shown to bind to intestinal mucus and mucin will be discussed (Table 1.1).

Table 1.1: Functionally characterised adhesins of *Lactobacillus* spp., which have been shown to adhere to intestinal mucus or mucin.

Adhesin	<i>Lactobacillus</i> strain	Target	Reference
<i>LPXTG-motif proteins</i>			
Mub	<i>L. reuteri</i> 1063	Hen intestinal mucus, pig mucin	Roos and Jonsson, 2002
Msa	<i>L. plantarum</i> WCFS1	Mucus via mannose residues	Pretzer <i>et al.</i> , 2005
<i>Anchorless housekeeping proteins</i>			
EF-Tu	<i>L. johnsonii</i> La1 NCC 533	Human intestinal epithelial cell line, mucin	Granato <i>et al.</i> , 2004
GroEL	<i>L. johnsonii</i> La1 NCC 533	Human intestinal epithelial cell line, mucin	Bergonzelli <i>et al.</i> , 2006
<i>Transporter proteins</i>			
MapA	<i>L. reuteri</i> 104R	Porcine intestinal mucus, human intestinal epithelial cell line	Rojas <i>et al.</i> , 2002; Miyoshi <i>et al.</i> , 2006

1.5.1.4.1. LPXTG – Motif Proteins

LPXTG motifs, found on the C-terminus of some proteins are used for attachment to the cell envelope using sortase dependent anchoring (Antikainen *et al.*, 2009). These proteins are targeted to the cell membrane by an N-terminal signal peptide, and a membrane-associated sortase enzyme cleaves off the LPXTG motif which allows the protein to link to the peptidoglycan peptide crosslinks by means of a covalent bond (Paterson and Mitchell, 2004).

Two LPXTG – motif proteins have been shown to function in adhesion to intestinal mucus, or mucus components, Mub from *L. reuteri* 1063 and Msa from *L. plantarum* WCFS1 (Vélez *et al.*, 2007).

Mub is a surface protein isolated from *L. reuteri* 1063 by functional screening of clones for adhesion of Caco-2 cells and intestinal mucus (Roos and Jonsson, 2002). It has since been shown, using genome sequencing, that several *Lactobacillus* spp. contain similar proteins. Mub domains within these proteins are of variable sequence and length, and occur in varying number of repeats, as shown in Figure 1.5 (Boekhorst *et al.*, 2006). It has been hypothesised that *Lactobacillus mucosae* was the original source of the *mub* genes (Roos *et al.*, 2000).

The mannose specific adhesin (Msa) was identified as the protein responsible for adhesion of *L. plantarum* strains to the mannose residues of *Saccharomyces cerevisiae*. The function of the *msa* gene was confirmed by the functional analysis of a deletion mutant (Pretzer *et al.*, 2005). Porcine intestinal tissue was perfused with *L. plantarum* 299v and an *msa* mutant of the same strain *in situ*, and it was shown that more wild type than *msa* mutants were bound to the intestinal surface. This suggests that *msa* confers a competitive advantage in adhesion to the intestinal surface (Gross *et al.*, 2008). After the identification of Msa as a sortase dependent adhesin, it was shown to have Mub domains, and was one of the genes identified by Boekhorst *et al.* (2006) in their screen for Mub domain-containing proteins. Comparison

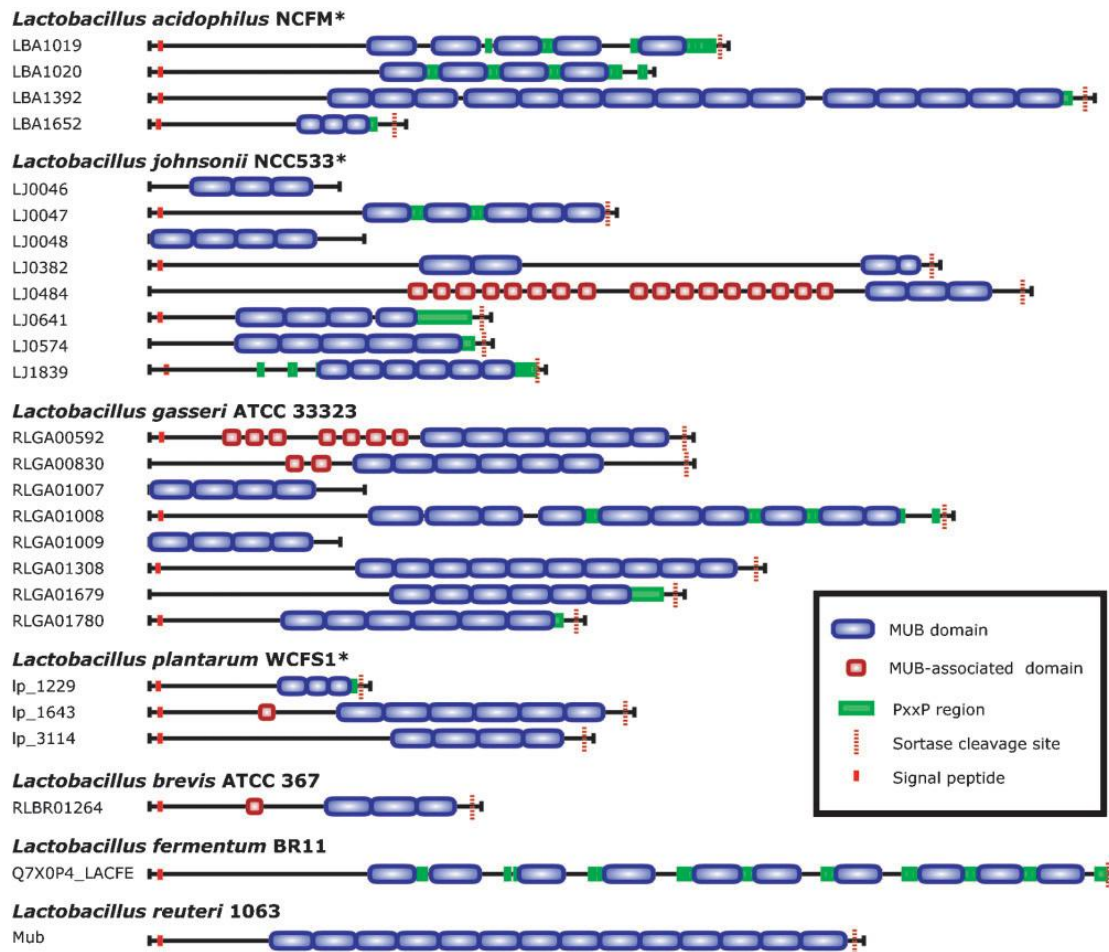


Figure 1.5: Domain composition of genes of *Lactobacillus* spp. containing three or more MUB domains. Modified from Boekhorst *et al.* (2006).

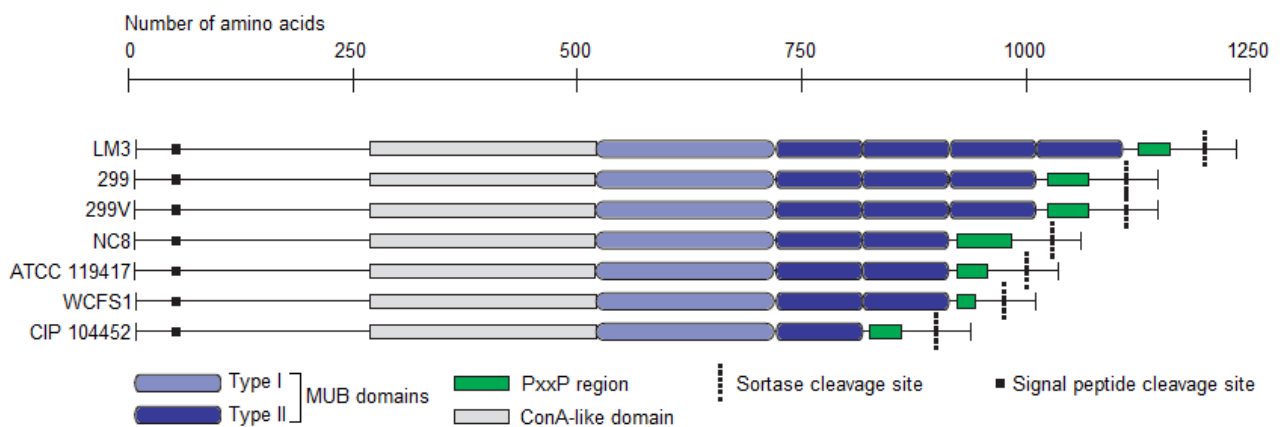


Figure 1.6: Domain composition of *msa* genes of *Lactobacillus plantarum* strains, highlighting the variation in numbers of repeating MUB domains. Modified from Gross *et al.* (2010).

of *msa* genes from different *Lactobacillus plantarum* strains has shown the variation in numbers of repeating MUB domains, even across members of the same species (Figure 1.6).

1.5.1.4.2. Anchorless Housekeeping Proteins

These surface proteins are referred to as “anchorless” as they have no known signal peptide sequences or membrane anchoring motifs in their predicted protein sequences (Antikainen *et al.*, 2009).

GroEL is a chaperone, which functions in intracellular folding of proteins. This protein has been localised to the cell envelope of probiotic strain *Lactobacillus johnsonii* La1 (NCC 533), using an ELISA with rabbit anti-GroEL antibody, and to the surrounding culture medium using a western blot with the same antibody. Recombinant GroEL, containing a His tag, has been shown to adhere to mucin derived from a human cell line (Bergonzelli *et al.*, 2006).

Elongation factor Tu (EF-Tu) is involved in intracellular protein synthesis. This protein was localised to the external surface of *Lactobacillus johnsonii* La1 (NCC 533) by extracting the outer surface proteins and subjecting this to western blotting using anti-EF-Tu antibody. Using similar methods to those outlined above, this recombinant protein was shown to adhere to human mucins (Granato *et al.*, 2004).

It has been suggested that GroEL and EF-Tu are implicated in adhesion of *L. johnsonii* La1 (NCC 533) to intestinal surfaces due to the adhesion of recombinant forms of these proteins to intestinal epithelial cells and mucus. Neither of GroEL, nor EF-Tu have been shown to contain protein domains or motifs which would explain the translocation of these proteins across the cell membrane (Bergonzelli *et al.*, 2006; Granato *et al.*, 2004). The mechanism of action of these two proteins in their role of adhesion has not yet been investigated. Unfortunately, the essential function of these genes in other cell functions prevents the confirmation of these adhesion functions using deletion mutants.

1.5.1.4.3. Transporter Proteins

There are several adhesins whose protein sequences show homology to the amino acid binding subunit of an ABC type transporter. One of these is the mucus adhesion promoting protein (MapA). This was isolated from *Lactobacillus reuteri* 104R and shown to adhere to mucus and mucin (Rojas *et al.*, 2002). MapA was later shown to be involved in the binding of *L. reuteri* 104R to human intestinal epithelial cells (Miyoshi *et al.*, 2006). A deletion *mapA* mutant has been shown to have reduced persistence in the GIT of mice *in vivo* (Havenith *et al.*, 2002). As a result of the sequence similarity of *mapA* to cysteine transporters and the similarity of the proposed operon organisation of *mapA* to that of *bspA*, a known cysteine transporter, it has been suggested that MapA also functions as a cysteine transporter. No other adhesins have been shown to function as amino acid transporters (Havenith *et al.*, 2002), and this has not yet been confirmed experimentally.

1.6.Delivery Mechanisms for Probiotics

Due to the difficulties associated with identifying an effective probiotic, some poultry farmers apply a “competitive exclusion” treatment to their chicks, which consists of administering a saline suspension of the contents of the alimentary canal from healthy adult birds to chicks. This is an effective treatment; however, due to the nature of the inoculum, extensive screening for human and avian pathogens must be done. Using a mixed culture of known probiotics eliminates the need for this expensive screening process (Mead, 2000). It has also been suggested that competitive exclusion treatments which are already known to be effective in improving the health of chicks should have their constituents identified and characterised. This would prevent the use of competitive exclusion treatments containing pathogens, or transferable virulence factors and antibiotic resistance determinants (Wagner, 2006).

There are some commercially available competitive exclusion treatments for use in chickens, such as Broilact (Orion Corporation) and Aviguard (Bayer AG). These products were found to be effective in the control of salmonella infection, and were partially characterised (Mead, 2000; Nakamura *et al.*, 2002).

There are many methods used to administer probiotics to poultry. The two most common are the addition of a probiotic suspension in the drinking water soon after birth, and the inclusion of a freeze dried preparation in the feed (Timmerman *et al.*, 2006). When the probiotic is intended to prevent colonisation by a pathogen such as *Salmonella* spp., it would be useful if the chicks could be treated soon after birth. This can be achieved by spraying the chicks with a suspension of the probiotic. The chicks preen, taking in the droplets of probiotic suspension orally. “Competitive exclusion” treatments have been known to be injected into the egg shortly before hatching. This, theoretically, would allow probiotic treatment before any pathogenic infection could occur. However, many eggs were unable to hatch after this treatment, due to the gas production or proteolytic action of some of the microorganisms (Mead, 2000). Timmerman *et al.* (2006) found that using drinking water as a delivery mechanism did benefit the treated chicks, but further research is required to determine if it is as effective as freeze dried preparations.

1.7.Probiotics Used in Poultry

Strains used as probiotics in general include *Bacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *E. coli*, *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp. and some yeast species (Patterson and Burkholder, 2003). Two of the many probiotic strains which have been identified and are currently in use in poultry are *Lactobacillus salivarius* CTC2197, *Enterococcus faecium* NCIB 10415, *Lactobacillus* sp. No I-2673 and *Lactobacillus johnsonii* FI9875.

Pascual *et al.* (1999) found that *L. salivarius* CTC2197 was appropriate for use in chickens, particularly in the prevention of *Salmonella* spp. infections. The survival of this probiotic was improved by the administration of more than one dose. When *E. faecium* NCIB 10415 was used in a feeding trial with turkey poults, it was found that the production of acid by this strain encourages the growth of lactic acid bacteria. The resulting production of lactate allows lactic acid bacteria to outcompete other microorganisms, including pathogenic bacteria (Vahjen *et al.*, 2002). *Lactobacillus* sp. No I-2673 reduced the colonisation of chickens by *Clostridium perfringens*, but had no effect on the lactobacilli or coliform numbers in the caecum (Gérard *et al.*, 2008). Colonisation of White Leghorn chicks by *Clostridium perfringens* was reduced by treatment with probiotic strain *Lactobacillus johnsonii* FI9875 (La Ragione *et al.*, 2004).

1.8.This Study

While sequencing of the caecum contents of an adult ostrich has provided some insight into the composition of the microbiota of an ostrich, very little research has explored the culturable microbiota of ostriches (Matsui *et al.*, 2010a). However, the strategy of probiotic selection and *in vivo* testing outlined here should succeed in identifying a probiotic that will improve the health of young ostriches, despite the fact that little is known about the microbiota of ostriches, and nothing is known about probiotic strains specific to ostriches.

In previous work, strains were selected from the GIT of adult ostriches, both wild and farmed, which did not receive antibiotic treatment (Reid, 2010). These strains were tested for characteristics of probiotics (Juste-Poinapen, 2007), and five strains were selected for further study (Table 1.2). Four of the five strains have been identified conclusively. The characterisation of these strains has included the acid and bile tolerance, adhesion to yeast, autoaggregation, production of antimicrobial compounds, production of enzymes, and survival of packaging methods commonly used for probiotics. The adhesion of the strains to

Table 1.2: Identities and origins of the five selected strains used in this study.

Strain	Origin	Identity
5934.3.1	Farmed	<i>Lactobacillus oris</i>
512.3.1	Farmed	<i>Lactobacillus brevis</i>
136.2.2J	Farmed	<i>Lactobacillus johnsonii</i>
136.3.1	Farmed	<i>Bifidobacterium pseudolongum</i> subsp. <i>globosum</i>
P1.2	Wild	n/c

Farmed: Isolated from the GIT of farmed ostriches.

Wild: Isolated from the GIT of a wild adult ostrich.

n/c: Strain has not been conclusively identified.

host epithelium and modulation of host immune system has not been examined, as there is little genetic information available about the ostrich immune system, and there is no available ostrich epithelial cell line.

Table 1.3 summarises the characterisation of the five selected strains. All of the strains were able to adhere to yeast and autoaggregate to some degree, and all strains showed some tolerance to acidic conditions or bile salts. Three of the strains were found to inhibit the growth of indicator strains, including clostridia, which may contribute to enteritis in ostrich chicks. The five selected strains also all produce at least two extracellular enzymes, which may aid in nutrition by degrading indigestible compounds and releasing products which are digestible by the host. Juste-Poinapen (2007) compared these characteristics to those of other

Table 1.3: Probiotic characteristics of the five selected strains used in this study. Modified from Juste-Poinapen (2007).

Strain	Probiotic Characteristics					
	Yeast Agglutination ^a	Auto-aggregation ^b	Antimicrobial Action ^c	Acid Tolerance ^d	Bile Tolerance ^e	Enzyme Production ^f
5934.3.1	+	++	<i>S. typh</i> , <i>E. faec</i> , <i>C. bot</i>	R	R	cell, pect, phyt, prot
512.3.1	++	+	<i>S. typh</i> , <i>S. aur</i> , <i>C. bot</i>	R	R	cell, xyl
136.2.2J	+++	+	(n/c mixed strain)	R	R	amyl, cell, prot
136.3.1	++	++	none	S	R	amyl, prot
P1.2	++	+	<i>E. coli</i> , <i>S. typh</i> , <i>C. perf</i> , <i>C. bot</i>	R	R	amyl, cell, pect, phyt, prot

a: + symbols indicate increasing agglutination, as defined by size of cell aggregates determined by microscopy

b: + indicates autoaggregation within 2 hours; ++ indicates autoaggregation within 10 minutes

c: Inhibition of growth of indicator strains *Escherichia coli* ATCC 25922 (*E. coli*), *Salmonella typhimurium* (*S. typh*), *Staphylococcus aureus* (*S. aur*), *Enterococcus faecium* van^S (*E. faec*), *Clostridium perfringens* (*C. perf*) or *Clostridium botulinum* (*C. bot*).

d: S indicates sensitivity; R indicates resistance or ability to grow at a pH of 3.

e: S indicates sensitivity; R indicates resistance or ability to grow in the presence of 1 % ox bile.

f: Production of amylase (amyl), cellulase (cell), pectinase (pect), phytase (phyt), protease (prot) or xylanase (xyl).

strains isolated from ostriches, and determined that these strains would be the most suitable candidates for further inclusion in the pilot feeding trials included in the same study.

The general aim of this work was to identify probiotic strains for use in ostriches, in order to improve the immune system of ostrich chicks to improve the health, perhaps reducing the high mortality rate and increasing the early weight gain.

The aims of this work were:

- ♦ to conclusively identify the last of the five selected strains;
- ♦ to confirm that the aggregation abilities demonstrated by the five strains in previous work indicate an ability to bind to intestinal mucin;
- ♦ to determine the effect of treatment with the five selected strains on mortality and weight gain of ostrich chicks;
- ♦ to monitor the development of the gut microflora over nine weeks;
- ♦ and to attempt to detect the persistence, or presence of the five selected strains in the faeces of treated birds using culture-based and culture independent techniques.

CHAPTER 2

IDENTIFICATION AND CHARACTERISATION OF STRAINS AND SCREENING FOR MUCIN ADHESION GENES

2.0.	Summary	30
2.1.	Introduction.....	31
2.2.	Methods and Materials.....	34
2.3.	Results.....	40
2.3.1.	Identification of Isolates	40
2.3.2.	Mucin Adhesion of All Strains	42
2.3.3.	Screening for <i>mapA</i> and <i>msa</i> Genes Using PCR.....	44
2.3.4.	Southern Hybridisation	48
2.4.	Discussion	49
2.4.1.	Identification of Isolates	49
2.4.2.	Mucin Adhesion of All Strains	50
2.4.3.	Screening for <i>mapA</i> and <i>msa</i> genes Using PCR.....	51
2.4.4.	Southern Hybridisation	53

2.0.Summary

Two of the five selected strains were conclusively identified using API testing and sequencing of the 16S rRNA gene and 16S – 23S intergenic spacer region. The ability of four of the five strains to adhere to mucin was determined using a standardised method. Two of the strains, *L. brevis* 512.3.1 and *L. oris* 5934.3.1 were shown to have the highest adhesion to mucin of all the selected strains. These two strains were screened for the *mapA* and *msa* genes using PCR, which have a role in adhesion to mucin. Southern hybridisation of strain *L. brevis* 512.3.1 showed the absence of a putative *L. brevis msa* sequence.

2.1.Introduction

The five selected strains used in this study were previously characterised in order to determine their potential as probiotics. To this end, their tolerance of acid and bile, ability to agglutinate in the presence of mannose and to co-aggregate, their antimicrobial action against various indicator strains, antibiotic resistance and survival through various encapsulation methods, such as freeze drying and encapsulation in alginate, was examined. It was concluded that these five strains exhibit *in vitro* traits which may allow them to colonise the GIT, as discussed in chapter one (Juste-Poinapen, 2007).

It is important not only to identify the selected strains to species level, but also to be able to differentiate strains. This could be achieved via the identification of a sequence region unique to this strain, and would enable the tracking of these strains during feeding trails and during production.

Previous research identified four of the five probiotic strains used in this study, but the fifth strain, P1.2, was not conclusively identified (Juste-Poinapen, 2007). Here, a polyphasic approach has been used to confirm the identity of P1.2. This approach entails the use of both physiological characteristics and sequencing of conserved genes. The strains were identified via a series of physiological tests, including substrate utilisation, in the form of an API kit. API testing is the most standardised and efficient way of performing these physiological assays, but they are often only able to identify the strain to genus level, due to several species having similar physiological profiles. Thus, conserved gene sequencing is employed in order to definitively characterise the species.

In this case, the sequences of two genes were used, namely the 16S rRNA gene, and the 16S – 23S intergenic spacer region (ITS). The 16S rRNA gene is the most conserved gene amongst bacteria and is routinely used as a marker. However, this gene is so highly

conserved among the lactic acid bacteria, that another, less conserved region, is used to identify the bacterium to species level (Tannock *et al.*, 1999). The sequence of the ITS may also provide a unique sequence tag, which could be used to differentiate an isolate from other lactobacilli strains.

Efficient colonisation of the gut by probiotic strains is of critical importance and the ability of the five strains to adhere to mannose and autoaggregate was previously investigated. Mannose residues line the GIT in the form of mucin glycoproteins (Kirjavainen *et al.*, 1998). Adhesion to mannose may potentially be linked to the ability of a strain to colonise the GIT, and previous studies have shown a link between autoaggregation and adhesion ability in some bifidobacteria (Collado *et al.*, 2007). While the previous assays may be an indicator of binding to the GIT, no direct assay of binding to intestinal mucin has been performed for these strains. A variety of methods used for this purpose has been reviewed by Vesterlund *et al.* (2005), all of which involve immobilising intestinal mucus onto a flat surface, and measuring the binding of the bacterial strain to this mucus layer after several washes. Methods used to measure binding include radioactivity, fluorescence, 4',6-diamidino-2-phenylindole (DAPI) staining and crystal violet staining. All of these methods rely on the availability of intestinal mucus. Other available methods for measuring the ability to bind to the GIT wall involve binding the bacterial strain to human cell lines (Tuomola and Salminen, 1998; Coconnier *et al.*, 1992), and are consequently not appropriate for the purposes of this study. A recent publication by Van den Abbeele *et al.* (2009) has described a standardised method for assaying binding to mucin. The methods described require binding of the bacterial strain to mucin agar, followed by washing off of excess bacteria from the agar, and finally, a release step, after which the bacteria are enumerated.

This method describes mucin agar prepared from commercially available, partially purified porcine mucin. There is no similar mucin preparation available from ostriches. Nevertheless,

a study by Rinkinen *et al.* (2003) has shown that the ability to bind to intestinal mucus is dependent on the bacterial strains, and not on the source of mucus. Therefore a strain that binds well to mucus from one animal species will bind well to mucus from another species. For this reason, commercially available porcine mucin could be used for the purposes of this study.

As discussed in chapter one, of all the genes implicated in bacterial adhesion to the mucin, *mapA* and *msa* are the best candidates for their involvement in adhesion of the selected strains to mucin (Vélez *et al.*, 2007). These genes were screened for using PCR primers designed to various regions of these genes, and in one case, using Southern hybridisation.

The aims of this chapter were to conclusively identify strains P1.2 and 5934.3.1 and to complete the characterisation of the selected strains by assaying their adhesion capability to mucin. In addition, the presence of the *mapA* or *msa* genes in the selected strains was to be determined. If these genes were indeed present, then the variability found among previously sequenced MUB domain-containing genes, discussed in chapter one, suggested that there may be some sequence features which were unique to this strain, which could be used to identify the probiotic strains during feeding trials.

2.2.Methods and Materials

Bacterial Strains and Culture Conditions: Five strains selected previously were used in this study. Strains *Lactobacillus oris* 5934.3.1, *Lactobacillus brevis* 512.3.1, *Lactobacillus johnsonii* 136.2.2J and *Bifidobacterium pseudolongum* subsp. *globosum* 136.3.1 were originally isolated from the GIT of farmed ostriches, which did not receive antibiotics, while strain P1.2 was isolated from the GIT of an adult wild ostrich (Reid, 2010). *Lactobacillus plantarum* P1.1.2.2 was originally isolated from the GIT of a wild adult ostrich, and was used as a positive control for the *L. plantarum* Msa PCR. *Lactobacillus rhamnosus* GG was used as a positive control for mucin adhesion studies (Van den Abbeele *et al.*, 2009). *Lactobacillus reuteri* ATCC 23272^T was used as a positive control for the *L. reuteri* MapA PCR. Cultures were grown anaerobically in a Forma Scientific anaerobic system (model 1024) in a gas mixture of 5% hydrogen, 10% carbon dioxide and 85% nitrogen. The selected strains were cultured in de Man Rogosa Sharpe (MRS) broth (Merck), or agar (15g/l (Merck)). MRS media were supplemented with 0.05% cysteine hydrochloride (Sigma Aldrich).

Escherichia coli DH5 α was used as a cloning host for plasmids (Sambrook *et al.*, 2001). This strain, and strains derived from it were grown aerobically at 37°C in LB broth or agar (15 g/l (Merck)) with or without 100 μ g/ml ampicillin (Sigma Aldrich, sodium salt) as applicable.

Genomic DNA Extraction: Bacteria were prepared by anaerobic culture in 10 ml MRS broth for 17 hours. Purity of the culture was assessed by Gram stain. Six millilitres of bacteria were harvested by centrifugation (1 min, 10 000 rpm). The pellet was resuspended in 200 μ l lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH8, 2mM EDTA, 20 mg/ml lysozyme (Sigma-Aldrich)) (Kullin, 2010). The suspension was incubated at 37°C for 30 min, followed by addition of 0.1 mg/ml proteinase K (Fermentas Life Sciences) and incubation at 37°C for a further 30 min. This pretreated sample was used as starting material in the Genomic DNA Purification Kit (Fermentas Life Sciences) as per manufacturers' instructions, with one

Table 2.1: Primer sequences for polymerase chain reactions.

Target	Product Length (bp)	Primer		Reference
		Name	Sequence	
16S rRNA gene	1500	F27	5'-AGAGTTTGATCCTGGCTCAG-3'	Lane, 1991
		R5	5'-ACGGTTACCTTGTTACGACTT-3'	Wheeler Alm <i>et al.</i> , 1996
ITS	variable	16-1A	5'-GAATCGCTAGTAATCG-3'	Tannock <i>et al.</i> , 1999
		23-1B	5'-GGGTTCCTCCCATTCGGA-3'	
pTZ57R/T insert	variable	M13F	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'	Yanisch-Perron <i>et al.</i> , 1985
		M13R	5'-GAGCGGATAACAATTTTCACACAGG-3'	
Degenerate MapA	390	MapA-F	5'-TTTGAAGT(CA)GA(CT)CTGGTAAAGC-3'	Juste-Poinapen, 2007
		MapA-R	5'-C(CA)CG(TG)GAGTT(AG)AT(CT)GT(CT)CC-3'	
<i>L. reuteri</i> MapA	822	LRMapAF	5'-GGAATCACAAGTGTTTCTGC-3'	This study
		LRMapAR	5'-GATCCAATGGGAACCTTAGC-3'	
<i>L. plantarum</i> Msa	1036	1229-midF	5'-GGTAATTTAGGTATTGGTGG-3'	Gross <i>et al.</i> , 2010
		1229-midR	5'-TGACACATGCTGCAAATCTG-3'	
<i>L. brevis</i> Msa	615	LbMsaF	5'-AACTGCTGCAAGTTCTTAGC-3'	This study
		LbMsaR	5'-AATGGCTCCGCTACTTACG-3'	

modification. Prior to precipitation of DNA by ethanol, 0.5 µl RNase (Fermentas Life Sciences) was added, and samples were incubated at 37°C for 20 min. Concentration of genomic DNA was assessed using a Nanodrop ND-1000 (Thermo Scientific, U.S.A.). Quality of genomic DNA was assessed by gel electrophoresis on a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 0.5mM EDTA) at 5.5 V.cm⁻¹ for one hour. All gels were visualised using ethidium bromide under short wavelength UV light in a Gel Doc XR (Bio-Rad).

Cloning of 16S rRNA and 16S – 23S intergenic spacer region: Fragments were amplified by PCR. Reactions had a total volume of 50 µl and contained 1 U Kapa Taq DNA polymerase (Kapa Biosystems, R.S.A.), 1X buffer A (at 1.5 mM MgCl₂), 0.2mM dNTPs, 0.5 µM each primer (Table 2.1) and 200 ng genomic DNA. PCR was carried out using a GeneAmp PCR System 9700 (Applied Biosystems). All PCR cycling conditions had an initial denaturation step for 5 min, followed by cycles of denaturation and annealing for 30 s, described in detail in Table 2.2. These were followed by an elongation step at 72°C and a

Table 2.2: Variable PCR conditions used for detection of gene fragments.

Target	No. cycles	Temperature (°C)			Elongation time (s)
		Initial denaturation	Denaturation	Annealing	
16S rRNA gene	25	96	96	55	90
16S - 23S intergenic spacer region	30	94	95	55	30
pTZ57R/T insert	30	96	95	50	60
Degenerate MapA	30	95	95	53	30
<i>L. reuteri</i> MapA	30	95	95	48	60
<i>L. plantarum</i> Msa	30	95	95	49	90
<i>L. brevis</i> Msa	30	95	95	44-50	45

final elongation step at 72°C for 5 min. Amplicons were separated by electrophoresis at 5.5 V.cm⁻¹ for 90 min on 0.8% and 2% agarose gels (TAE) in order to confirm the sizes of the 16S rRNA gene and 16S – 23S intergenic spacer region amplicons respectively. The PCR product was purified using the Biospin PCR Purification kit (Bioflux, Japan) as per manufacturer's instructions. The purified amplicon was ligated into the vector pTZ57R/T as per the manufacturer's instructions (InsTAclone Cloning kit, Fermentas Life Sciences). Plasmids were then transformed into RbCl competent *E. coli* DH5α cells (Armitage *et al.*, 1988). Plasmids were extracted from transformants (Ish-Horowicz and Burke, 1981) and screened for insert of appropriate size using PCR amplification with M13 primers. Plasmids were then purified using a Biospin PCR Purification kit (Bioflux, Japan) as per manufacturer's instructions before being subjected to sequencing by Macrogen Inc (Korea).

Bioinformatic Analysis: Sequence data obtained were analysed using Chromas version 2.01 (Technelysium) and DNAMAN version 4.13 (Lynnon Biosoft), and compared to those found in the GenBank database using the BLAST algorithm (Altschul *et al.*, 1997). The phylogenetic tree was constructed using maximum parsimony and the close-neighbour-interchange (CNI) algorithm in MEGA version 4.0 (Tamura *et al.*, 2007). *Lactobacillus reuteri* MapA primers were designed to the sequence of the *mapA* gene of *L. reuteri* 104R

(accession number AJ293860) using standard methods. Msa primers were designed to the genome of *L. brevis* ATCC 367 using the tBLASTn algorithm (Altschul *et al.*, 1997) the Msa protein sequence as a query (accession number NP 784891) and standard methods.

API Tests: Strain P1.2 was subjected to a rapid ID 32 STREP API test (Biomérieux, France) as per manufacturer's instructions. Results were analysed using APILAB version 3.3.3.

Triton Survival Curve: Cells from five millilitres of a stationary phase (18 h) culture of the relevant strain were collected by centrifugation under anaerobic conditions and resuspended in 0.5 % Triton X-100 to 10^8 cells/ml. The OD₆₀₀ of a cell suspension required to give a titre of 10^8 cfu/ml was determined for each strain by dilution and plating. After 0, 30 and 60 min, 100 µl of each reaction was enumerated by dilution in phosphate buffered saline (PBS), pH 5.9, and plating on MRS.

Mucin Adhesion Assay: Assay was performed as described by (Van den Abbeele *et al.*, 2009). Briefly, 10^8 cells are added to 5 % (w/v) mucin agar, pH 6.8 (porcine mucin type II (Sigma), 8 g/l bacteriological agar (Merck)) in a 12 well microtitre plate (Corning Incorporated, U.S.A.). Bacterial suspensions were incubated with mucin agar anaerobically with shaking at 50 rpm for 80 min. During this incubation the original bacterial suspension was enumerated by dilution and plating on MRS agar. Each cell suspension was removed by pipetting and wells were washed twice with PBS, pH 5.9, in order to remove non-adhered cells. This was followed by the addition of 0.5 % Triton X-100 to each well to release the adhered cells. The 0.5 % Triton on the mucin agar were incubated anaerobically with shaking at 100 rpm for 20 min. The Triton X-100 was collected and each well was washed twice with PBS. These PBS washes were added to the collected Triton X-100 wash, and this was enumerated by dilution and plating. Each assay was done in technical and biological triplicate. Technical replicates were represented by replicate wells of mucin agar containing

the same culture, while biological replicates were represented by replicate cultures of the same strain.

PCR detection of gene fragments: All reactions had a total volume of 25 μ l, containing 0.5U Kapa Taq (Kapa Biosystems, South Africa), 0.2 mM dNTPs, 0.5 μ M each primer (Table 2.1), 1x buffer A (containing 1.5 mM MgCl_2) and 200 ng genomic DNA. For the PCR using LbMsa primers at low annealing temperatures, in order to confirm absence of amplicon, MgCl_2 concentration was adjusted to 4.5 mM. The plasmid pMapJ, previously constructed by (Juste-Poinapen, 2007) by ligating a fragment amplified by PCR using degenerate *mapA* primers from strain 512.3.1 into pTZ57R (Fermentas Life Sciences), was used as a positive control for the degenerate MapA primers. Amplification was carried out and amplicons were electrophoresed as described above.

Southern hybridisation: The presence of the *L. brevis msa* fragment was determined using Southern hybridisation of genomic DNA digested with the restriction endonuclease *Dra*I. DNA fragments were separated by electrophoresis on a 0.8 % agarose gel in 0.5x TBE buffer (pH 8.3, 44.5 mM Tris-borate, 1mM EDTA) at 1.25 V.cm^{-1} for 16 hours. The gel was stained in 1 μ g/ml ethidium bromide in TBE buffer for 30 minutes and destained in TBE buffer for 2 minutes. The gel was visualised under short wavelength UV light in a Gel Doc XR (Bio-Rad). DNA fragments were transferred to Hybond- N^+ membrane (Amersham Biosciences) using a Trans-Blot[®] SD Semi Dry Electrophoretic Transfer Cell (Bio-Rad) as per the manufacturer's recommendations. The probe was labelled by PCR using PCR DIG Probe Synthesis Kit (Roche), primers LbMsaF and LbMsaR and genomic DNA from *L. brevis* ATCC 14869^T as template. Hybridisation and stringency washes were carried out as described in DIG application manual for filter hybridisation (Roche Molecular Biochemicals), using a hybridisation temperature of 44°C and a high stringency wash at 68°C

in 0.1 x Saline Sodium Citrate buffer (15 mM NaCl, 1.5mM sodium citrate, pH 7.0) + 0.1% SDS.

2.3. Results

2.3.1. Identification of Isolates

Strain P1.2 was not conclusively identified previously, but its physiological characteristics showed it had potential as a probiotic. Therefore strain P1.2 was profiled in terms of its carbohydrate utilisation using API tests to establish its identity based on physiological markers. These tests revealed that strain P1.2 has a 99.9% similarity to *Enterococcus faecalis*. Table 2.3 shows the sequences from the GenBank database which had the highest homology to the 16S and ITS products from the isolates of interest. Strain P1.2 showed > 99% sequence homology to *Enterococcus faecalis* strains with regards to both gene marker regions.

Another strain, 5934.3.1, had been identified as *Lactobacillus oris* by preliminary API tests and sequencing of the 16S rRNA gene (Juste-Poinapen, 2007). Since there is high sequence similarity between the 16S sequences of *Lactobacillus* spp., the sequence of the 16S – 23S

Table 2.3: Sequences giving maximum identity to various cloned genes from isolated strains.

Strain	Gene	Most Significant Sequence Alignments				Query Coverage (%)
		Accession No.	Description	Maximum Identity (%)	E Value	
P1.2	16S	FJ378660.2	<i>Enterococcus faecalis</i> , strain HN-N5	100	0.0	99
		FJ378659.2	<i>Enterococcus faecalis</i> , strain HN-N4	100	0.0	99
		FJ378656.2	<i>Enterococcus faecalis</i> , strain HN-N1	100	0.0	99
		FJ378688.1	<i>Enterococcus faecalis</i> , strain HN-N33	100	0.0	99
		FJ378665.1	<i>Enterococcus faecalis</i> , strain HN-N10	100	0.0	99
	ITS	AE016830.1	<i>Enterococcus faecalis</i> V583	99	0.0	100
		AJ301841.1	<i>Enterococcus sulfureus</i> , strain LMG 13084	95	9.0x10 ⁻¹⁷⁶	88
		AJ301829.1	<i>Enterococcus dispar</i> , strain LMG 13521	96	9.0x10 ⁻¹⁶⁶	87
		AJ301839.1	<i>Enterococcus saccharolyticus</i> , strain LMG11427	96	5.0x10 ⁻¹⁶³	86
		AJ301826.1	<i>Enterococcus casseliflavus</i> , strain LMG 10745	95	3x10 ⁻¹⁴⁵	85
5934.3.1	ITS	EU191611.1	<i>Lactobacillus oris</i> , strain 353	99	0.0	99
		EF412990.1	<i>Lactobacillus panis</i> , strain C17	99	0.0	97
		EF412989.1	<i>Lactobacillus reuteri</i> , strain C16	100	0.0	94
		CP000705.1	<i>Lactobacillus reuteri</i> F275	100	0.0	97
		EF412988.1	<i>Lactobacillus reuteri</i> , strain C10	99	0.0	93

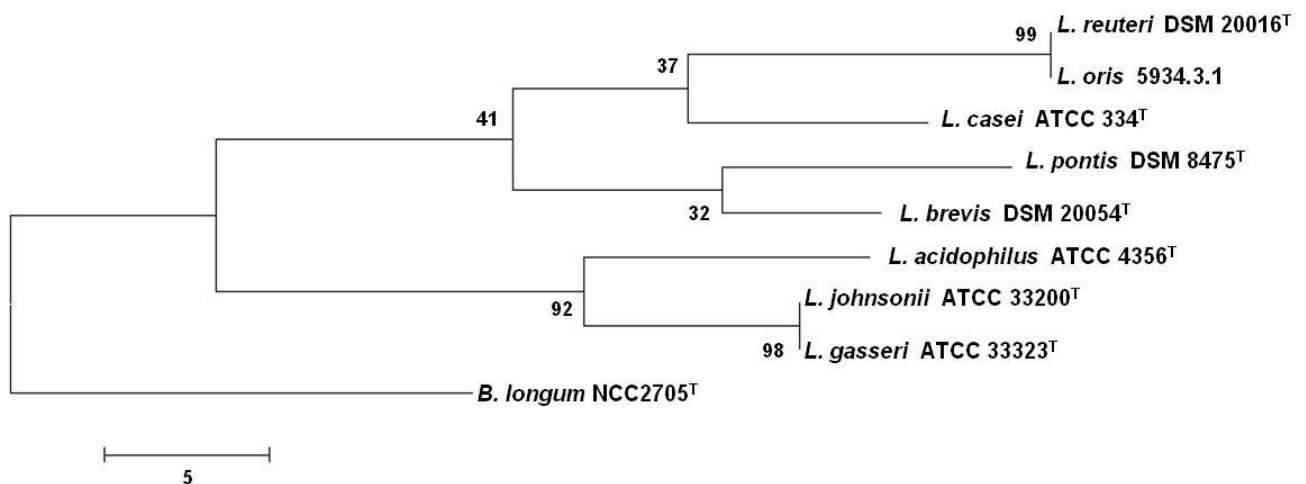


Figure 2.1: Phylogenetic tree constructed using the intergenic spacer regions of eight type strains and *L. oris* 5934.3.1, using maximum parsimony and the close neighbour interchange model. The alignment used for this analysis was 454 nucleotides long. Bootstrap values appear at the branch points (100 000 iterations). The accession numbers for the sequences used, excluding *L. oris* 5934.3.1, top to bottom, are: AF080100, AF121200, EU161607, AF405353, U32971, AF074860, AF074859 and GI58012118. The sequence of *B. longum* NCC 2705T was used to root the tree.

intergenic spacer sequence was used to confirm the identity of this strain. Strain 5934.3.1 showed 99% sequence homology to the 16S – 23S intergenic spacer region of *Lactobacillus oris* strain 353, which was isolated from the intestinal contents of a farmed ostrich, during the same study from which the five selected strains used in this work were isolated (Reid, 2010). This strain was identified as *L. oris* using methods similar to those presented here (Greenhill, 2007). There is no *L. oris* type strain ITS present in Table 2.3, because there is no sequence for this particular intergenic region, or complete genome sequence for this type strain present in the GenBank database. Phylogenetic analysis of the ITS regions of *L. oris* 5934.3.1 and eight type strains was completed in order to show the relatedness of these strains (Figure 2.1). This phylogenetic tree shows that *L. oris* 5934.3.1 is most closely related to *L. reuteri* DSM20016^T of the strains included in the analysis.

2.3.2. Mucin Adhesion of All Strains

The mucin adhesion assay requires that the strains used survive the presence of 0.5% Triton X-100 for the duration of the release and enumeration steps of the assay. This ensures that the measured values for adhesion will not be lower than the true values. Figure 2.2 shows the survival of the five probiotic strains and *L. rhamnosus* GG in 0.5% Triton X-100. Only 136.2.2J experiences a decrease of its cell titre, 1000 fold in 60 minutes, while the other five strains have a stable titre over the same period. Due to the loss of viability strain 136.2.2J was not assayed for mucin adhesion.

The mean percentage of cells bound to mucin for each strain is shown in Fig 2.3. Strain LGG gave the highest percentage of bound cells, followed by *L. brevis* 512.3.1 and *L. oris* 5934.3.1. The values for percentage adhesion ranged from ~ 0.2 - 1.4 %. Both P1.2 and 136.3.1 showed a percentage of bound cells that were significantly ($P < 0.05$) divergent from

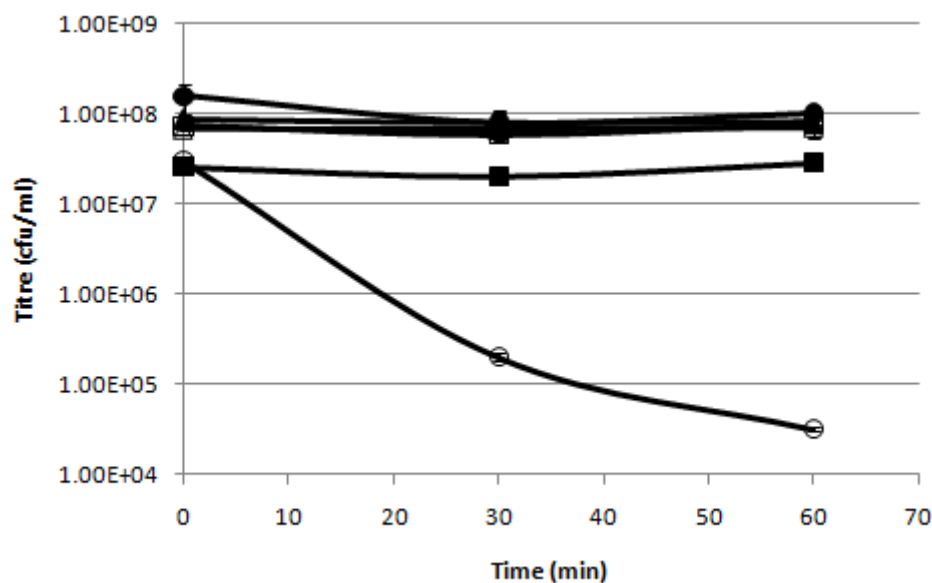


Figure 2.2: Survival after resuspension in 0.5% Triton X-100 of strains *L. rhamnosus* GG (■), P1.2 (▲), 136.3.1 (●), 512.3.1 (□), 5934.3.1 (Δ) and 136.2.2J (○). Error bars represent standard deviation of the mean (n=3).

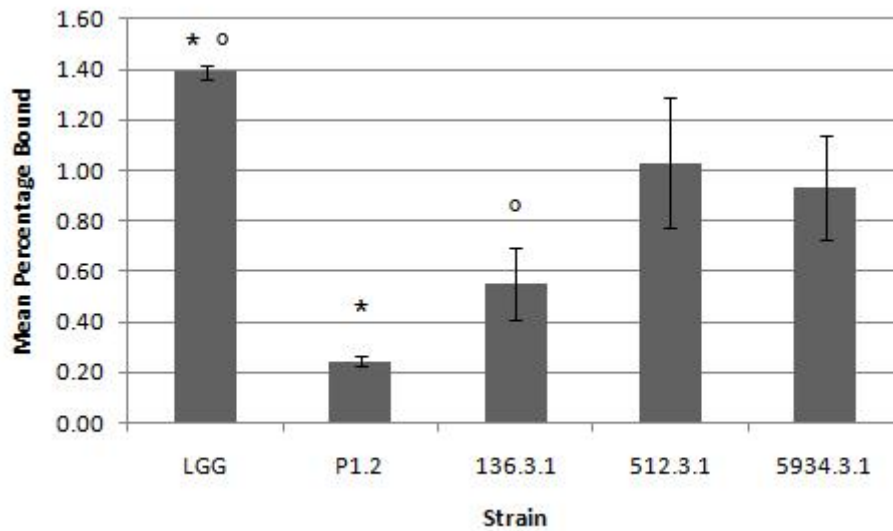


Figure 2.3: Binding of strains to mucin. LGG indicates *L. rhamnosus* GG. Error bars represent standard error (n=3). Student's t-test was used to calculate P values. Stars indicate a significant difference between strains LGG and P1.2 ($P=7.01 \times 10^{-6}$). Circles indicate a significant difference between strains LGG and 136.3.1 ($P=0.025$).

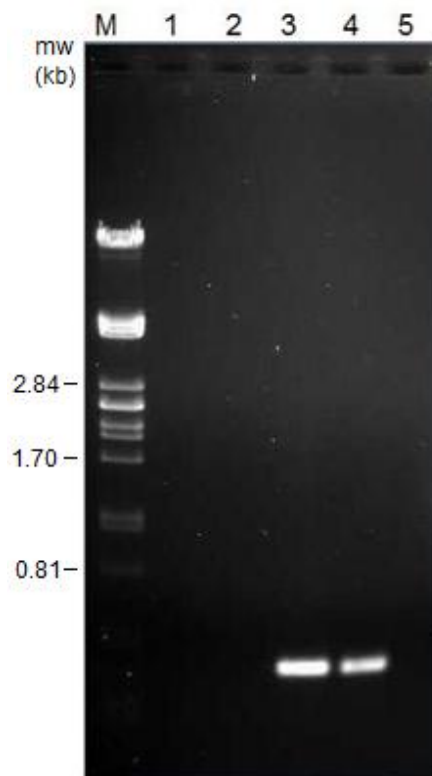


Figure 2.4: MapA fragment from PCR using degenerate MapA primers for strains 512.3.1 (lane 1) and 5934.3.1 (lane 2) pMapJ (lane 3) and strain *L. reuteri* ATCC 23272T (lane 4). Lane 5 shows the no template control. M represents a molecular weight marker of *Pst*I digested λ DNA. Mw (kb) indicates the molecular weight in kilobases of fragments in the adjacent lane.

that of LGG, while 512.3.1 and 5934.3.1 were not significantly different in their binding capabilities from *L. rhamnosus* GG.

2.3.3. Screening for *mapA* and *msa* Genes Using PCR

PCR was used to screen for the presence of genes known to play a role in adhesion to mucin, namely *mapA* and *msa*. Two sets of PCR primers were used to screen for each gene. Screening for *mapA* was limited to the strains which adhered to mucin best, strains *L. brevis* 512.3.1 and *L. oris* 5934.3.1. The first set of primers, the degenerate *mapA* primers, were designed to conserved nucleotide regions of the *mapA* gene (Juste-Poinapen, 2007). No *mapA* fragment could be amplified from strains 512.3.1 and 5934.3.1 using the degenerate *mapA* primers (Figure 2.4), even though the template DNA was successfully used to amplify the 16S rRNA gene, thus ensuring the integrity of this template DNA. However, fragments of expected sizes (390 bp) were obtained from pMapJ and *L. reuteri* ATCC 23272^T.

Specific primers were designed to the *mapA* gene of *L. reuteri* 104R, as this is the only annotated sequence of *mapA* available in GeneBank. These primers yielded a product from another strain of *L. reuteri*, namely *L. reuteri* ATCC 23272^T (Figure 2.5). This was expected, because MapA proteins from a subset of *L. reuteri* strains (including the type strain and strain 104R) have 98 % similarity at the amino acid level. PCR primers designed to the *mapA* gene of *L. reuteri* failed to amplify a *mapA* amplicon from strains 512.3.1 and 5934.3.1, as shown in Figure 2.5.

Attempts to amplify an *msa* fragment using primers designed to *L. plantarum* WCFS1 (Gross *et al.*, 2010) were unsuccessful for all five selected strains (Figure 2.6).

No published primers for the *msa* gene of other *Lactobacillus* spp. were available. Searching the GeneBank database using the Msa protein sequence as a query, and the tBLASTn algorithm showed that a region of the *L. brevis* ATCC 367 has some similarity to the Msa

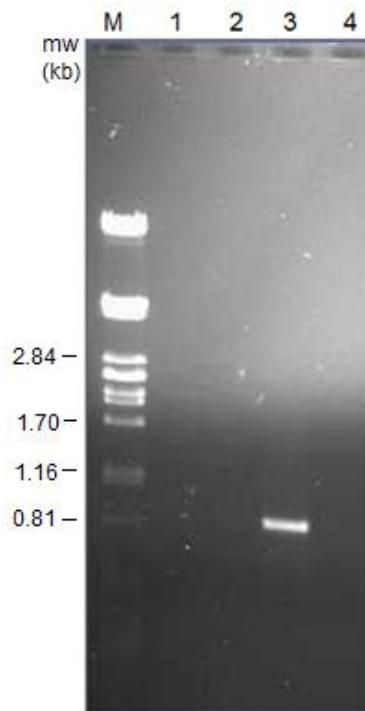


Figure 2.5: MapA fragment from PCR using primers designed to *L. reuteri* strain 104R for strains 512.3.1 (lane 1), 5934.3.1 (lane 2), and *L. reuteri* ATCC 23272T (lane 3). Lane 4 shows the no template control. M represents a molecular weight marker of *Pst*I digested λ DNA. Mw (kb) indicates the molecular weight in kilobases of fragments in the adjacent lane.

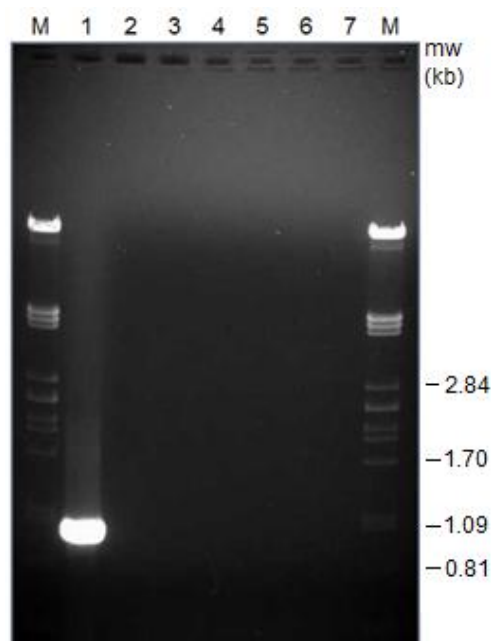


Figure 2.6: Msa amplicons from PCR using primers designed to *L. plantarum* WCFS1 for strains *L. plantarum* P1.1.2.2 (lane 1), 512.3.1 (lane 2), 5934.3.1 (lane 3), 136.2.2J (lane 4), 136.3.1 (lane 5) and P1.2 (lane 6). Lane 7 shows the no template control. M represents a molecular weight marker of *Pst*I digested λ DNA. Mw (kb) indicates the molecular weight in kilobases of fragments in the adjacent lane.

```

Lplan  261      LISDEAALTHVDKDNFLKYFSLNGSATYDAKTGIVTITPNQNNQVGNFSLTSKIDMNKSF 320
              + +  A  HVD+DNF +YF LNGSATYD TGIVT+TP++N+QVGNF+L  KID + +F
Lbrev  2233051  MATQRPAYVHVDQDNFTQYFDLNGSATYDKPTGIVTVPDKNDQVGNFALKPKIDASTNF 2232872

Lplan  321      TLTGQVNLG---SNPNGADGIGFAFHSGNTTDDVGNAGGNLGIGGLQDAIGFKLDTWFNSY 377
              TL  GQVNLG   S   GADGIGFAFH+GN+TD+GNAG NLGIGGL DA+G KLDTW N
Lbrev  2232871  TLLGQVNLGNRTSATGGADGIGFAFHNGNSTDIGNAGDNLGIGGLIDALGLKLDTWHNGA 2232692

Lplan  378      QAPSSDKNGSEISSTNSNGFGWNGDSANAPYGTfVKTsnQEISTANGSKVQRWWAQDTGE 437
              P + ++G+++S+T++NG+G   D   A +G FV T++Q+I   +G   RWWAQ
Lbrev  2232691  HMPEALRSGAQVSTTDANGYG*ANDPDPAQFGGFVTTTDDQIKAIDGHAYDRWWAQT--- 2232521

Lplan  438      SQALSKADIDGNFHDfVVNYDGATRtlTVsYtQASgKvLTWkTtVDSSyQAMAMVVSAST 497
              DF      + + L V+Y + +G VLTW      QA AMVVSAST
Lbrev  2232520  -----DF-----NSVQALQVTYEETNGHVLtWtKLAPVDRQAFAMVVSAST 2232398

Lplan  498      GAAKNLQQFKLTSFDFQEAATVNvKYVDtTGhQLAQGTANYPDGAYVNGRYTtTKQLiIPN 557
              G AKNLQQFKL SFDFQ+AAT+NV+YVD   G QLAQ T NYPDGA VNG Y T QL I
Lbrev  2232397  GGAKNLQQFKLESFDFQQAATMNvQYVDLNGRQLAQATVNYPDGAVVNGTYATsQLDISG 2232218

Lplan  558      YRFIKMDDGSVTGTksLDANGtLIQSGDNGtVIYVY--VPEYMAiVKtVnETiNYVDENG 615
              Y F+ M D   TG   S   ANG L + GDNGTVIYVY   P   A + V+E I+Y++E
Lbrev  2232217  YHFVNMGDNPTTGiss-PANGYLNRVGdNGtVIYVYDQNPVDYATTRVvHEMiHYLNEQQ 2232041

Lplan  616      HALTTSYtanPIHILtVTnPVdGtTTTTyStITtSiELdATTGRpVdSG--WVLGNSQD 672
              +   SY A PI   L V   P +G+ +TYYS      + L   T G+P+ +   W+ G +
Lbrev  2232040  QVIAPSYQAQPITFLQVKYPTNGSISTYySLGNNEVLLLNtAGQPLVtSTVRWIPGEAST 2231861

Lplan  673      FDAVTNPQIKGYtVtStDAPNSDLQHvSAQTVtGDsgDLEfTVVY 717
              F   VTNP ++GY   STDA NS+L   ++AQ VT   S DL+ TV Y
Lbrev  2231860  FSPVTNPVQGYQAVSTDALNSNLSEIAAQMVtTTSKDLDLTVYY 2231726

```

Figure 2.7: Sequence alignment of Msa protein from *L. plantarum* and translated genome of *L. brevis* ATCC 367, carried out using tBLASTn algorithm. The highlighted regions indicate conserved residues, to which the LbMsa primers were designed.

protein. This region had a maximum identity of 64 % with the Msa protein, 73 % query coverage, and an E value of 10^{-109} . The regions of conserved residues in an alignment between the *L. plantarum* Msa protein and the translated genome sequence of *L. brevis* ATCC 367 were used to design specific primers to the *L. brevis msa* gene (Figure 2.7).

Two different annealing temperatures were used for PCR with the LbMsa primers, the optimal annealing temperature (50°C) and a low annealing temperature (44°C), to determine the presence of amplicons under both PCR conditions. No amplicon was obtained when using primers designed to the *msa* gene of *L. brevis* and an annealing temperature of 50°C (Figure 2.8A). These primers yield some non specific amplicons when an annealing temperature of

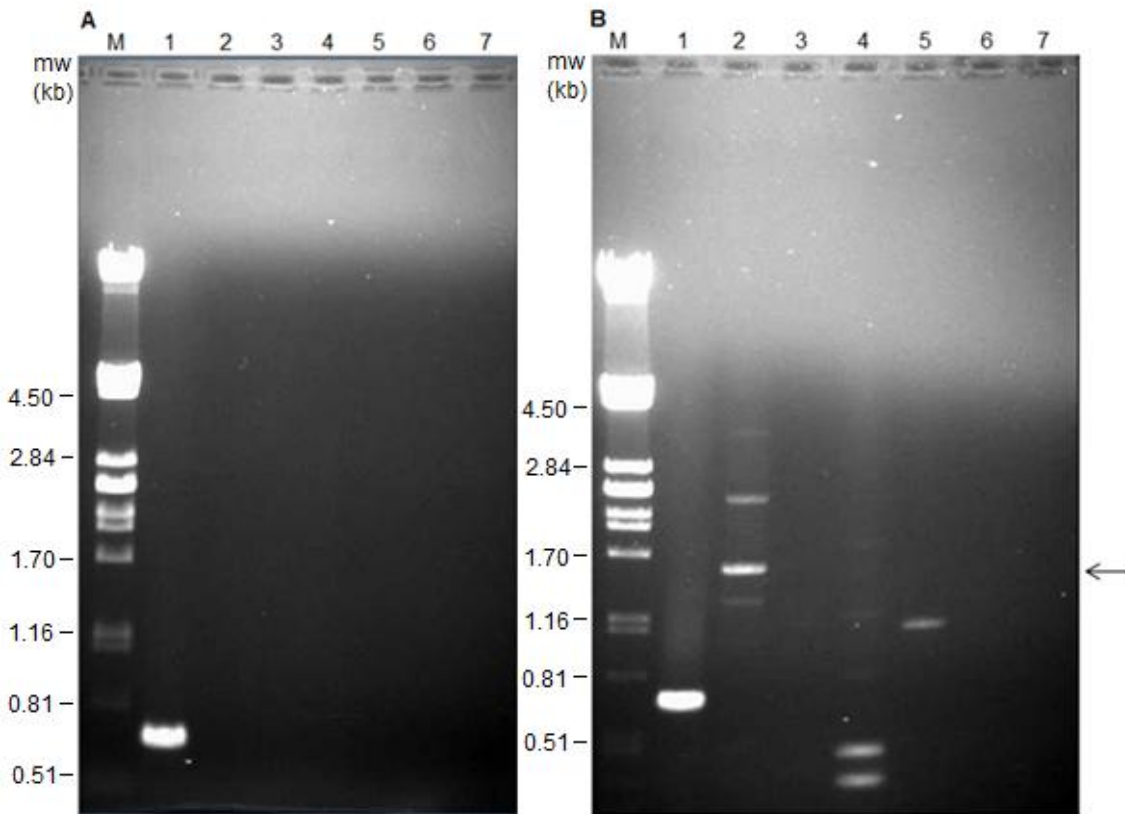


Figure 2.8: Msa amplicons from PCR using primers designed to *L. brevis* and an annealing temperature of (A) 50°C or (B) 44°C, from strains *L. brevis* ATCC 14869T (lane1), 512.3.1 (lane 2), P1.2 (lane 3), 136.2.2J (lane 4), 136.3.1 (lane 5) and 5934.3.1 (lane 6). The no template control is shown in lane 7. M represents a molecular weight marker of *Pst*I digested λ DNA. Arrow indicates the 1.4 kb amplicon in lane 2. Mw (kb) indicates the molecular weight in kilobases of fragments in the adjacent lane.

44°C is used, as shown in Figure 2.8B. The most abundant amplicon of strain 512.3.1, a ~ 1.4 kb amplicon indicated by the arrow in Figure 2.8B, was cloned and sequenced. Comparing the sequence of this fragment to the GenBank database using the BLAST algorithm revealed a maximum identity of 96 % to the genome sequence of *L. brevis* ATCC 367 (accession number CP000416.1). The E value was 0.0 and the query coverage was 99%. This region of the genome corresponds to a dipeptidase. However, this identity was between nucleotides 280170 to 281615, while the LbMsa primers were designed to bind between nucleotides 2232041 and 2233051.

2.3.4. Southern Hybridisation

Southern hybridisation was used to detect the presence of an *msa* fragment in strain *L. brevis* 512.3.1, since this strain is the most closely related to *L. brevis* ATCC 14869^T, to which the LbMsa primers were designed. Hybridisation of an *L. brevis* ATCC 14869T *msa* probe to *Dra*I digested genomic DNA from *L. brevis* ATCC 14869T showed the presence of a 2.4 kb *msa*-containing fragment in this strain (Figure 2.9). No fragment, of similar size or otherwise, is seen in strain 512.3.1.

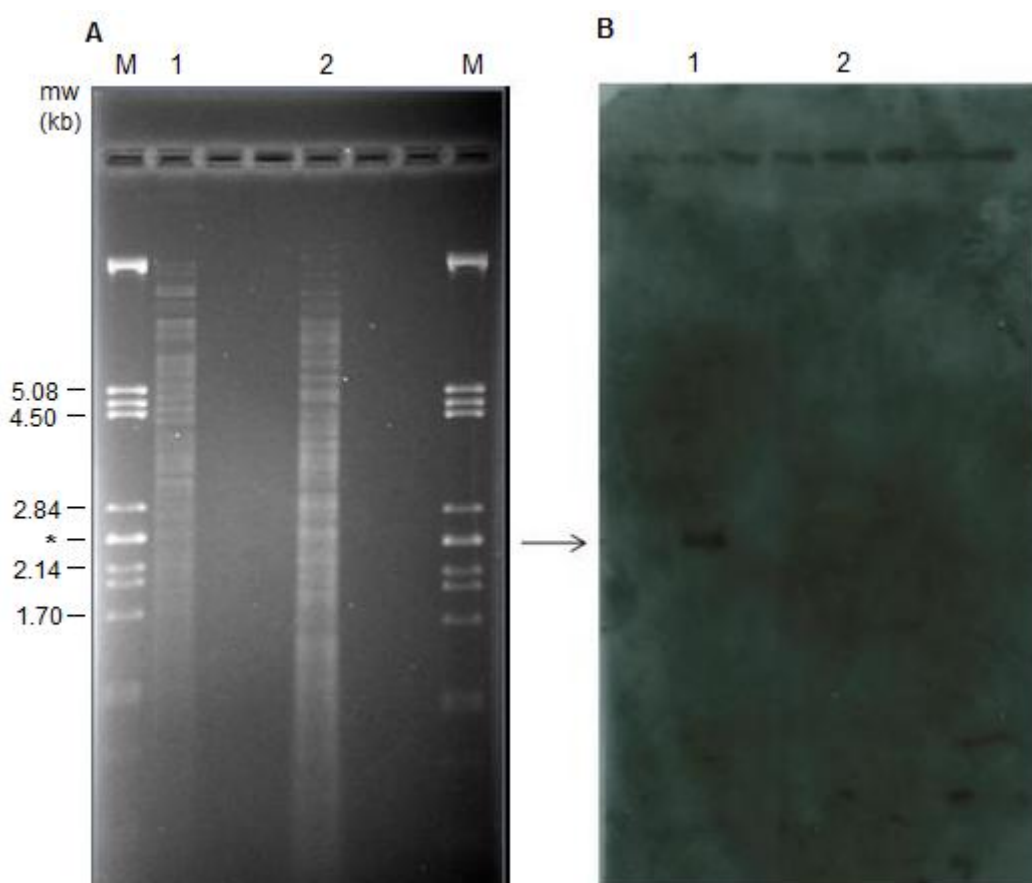


Figure 2.9: Southern hybridisation of *L. brevis* ATCC 14869T *msa* fragment to *Dra*I digested gDNA from *L. brevis* ATCC 14869T (lane 1) and 512.3.1 (lane 2). Panel A shows agarose gel before electrotransfer and panel B shows visualisation of hybridised membrane. M represents a molecular weight marker of *Pst*I digested λ DNA. Mw (kb) indicates the molecular weight in kilobases of fragments in the adjacent lane. Asterisk indicates a doublet of two fragments, 2,459 kb and 2,443 kb. Arrow indicates 2.4 kb band in lane 1.

2.4.Discussion

2.4.1. Identification of Isolates

Previous work gave ambiguous results as to the identity of strain P1.2 (Juste-Poinapen, 2007). This strain was initially identified as *Lactobacillus paracasei* via API testing (99.3% identity). The subsequent sequence of the 16S rRNA gene had 99% identity to *Lactobacillus reuteri*. Amplification of the ITS region was attempted, but failed to yield amplicons. However, the physiological properties of P1.2 indicated that it may have some potential as a probiotic, and its value as a probiotic warranted its inclusion in feeding trials. Therefore, this work was repeated in this study in order to confirm the identity of the strain before it could be subjected to *in vivo* testing.

The results of the API test obtained in this study indicate that P1.2 has 99.9% similarity to *Enterococcus faecalis*. This concurs with the results obtained by sequencing both the 16S rRNA gene and 16S – 23S intergenic spacer region. These also showed > 99% sequence homology to sequences from *Enterococcus faecalis* in the NCBI database.

Enterococcus species have been found in the GIT of other healthy poultry, especially those treated with antibiotics (Zhou *et al.*, 2007) and it has been suggested that this may be increased by treatment with antibiotics to which these bacteria are resistant. The bacteria which have been displaced by the antibiotic treatment may leave a niche which can be colonised by resistant enterococci (Wise and Siragusa, 2007). Some *Enterococcus* spp. have been found to be effective probiotics when administered alone (Samli *et al.*, 2007), or in a mixture with lactobacilli and bifidobacteria (Mountzouris *et al.*, 2007). This genus is considered to be a member of the lactic acid bacteria, which, as a group, are present in many foods and many members are generally regarded as safe (GRAS). However, the presence of enterococci in infection sites and the antibiotic resistance of some species have led to a

requirement for *Enterococcus* spp. to be checked for safety before they can be released as feed supplements in the European Union (Becquet, 2003). This safety assessment includes screening for transferable antibiotic resistance elements. However, there are *Enterococcus* strains that have been approved for use in livestock, such as poultry (Jennes *et al.*, 2000), and in humans (Domann *et al.*, 2007).

The identity of strain 5934.3.1, previously shown to be *L. oris* using API testing and sequencing of the 16S rRNA gene, (Juste-Poinapen, 2007) was confirmed by sequencing of the ITS region. The sequence of the ITS region has 99% similarity to *L. oris* 353. A phylogenetic tree constructed using the ITS regions of *L. oris* 5934.3.1 and eight type strains showed that strain 5934.3.1 clustered with the *L. reuteri* type strain. No type strain sequence was used for *L. oris* ATCC 49062^T, as no ITS region sequence is available for this strain. *Lactobacillus* spp. are generally regarded as safe for consumption (Salminen *et al.*, 1998).

2.4.2. Mucin Adhesion of All Strains

The ability to interact with mucin is not found in all intestinal bacteria, but it has been confirmed to be important for successful colonisation of the intestinal tract by lactobacilli and other probiotic bacteria (Matsumoto *et al.*, 2002; Van den Abbeele *et al.*, 2009). Binding to mucin will be used as another characteristic for selecting probiotic strains for use in an *in vivo* trial.

The method used to assay the ability of the strains to bind to mucin requires that the strains be washed off the mucin agar using 0.5% Triton X-100 before the cell number is determined by dilution and plating. It was therefore necessary to show that the cells are able to survive the treatment with Triton X-100, which at higher concentrations, is a reagent that is used to lyse cells. If this was not shown, then it would be possible that the bound cells washed from the agar would be killed by the Triton X-100, and the assayed number of cells will be lower than

the true number of cells. The survival of the strains at this concentration was found to be stable for all strains except strain *L. johnsonii* 136.2.2J. For this reason, the ability of this strain to bind mucin could not be assayed using this method. Another protocol would have to be optimised to assay this strain, perhaps using a different detergent.

It is difficult to compare adhesion values across several studies, as strains bind differently according to the binding matrix used (Tallon *et al.*, 2007; Vélez *et al.*, 2007). Nevertheless, strain LGG was tested previously by the group who developed this method, and has been shown to bind well to intestinal mucus and human cell lines (Van den Abbeele *et al.*, 2009). The group found that this strain had 14.7 ± 3.1 % adhesion and it is therefore regarded as a positive control for this assay. This value is approximately 10 fold more than the percentage adhesion measured in this study for strain LGG. While the adhesion values for the five selected strains are lower than those reported for strains which have good adhesion capabilities, so is the value obtained for strain LGG. For this reason, the adhesion values for the five selected strains can only be compared to strain LGG (the positive control) and each other. Strains *L. brevis* 512.3.1 and *L. oris* 5934.3.1 have the best adhesion capabilities of the assayed probiotic strains.

Adhesion to mucin may be a mechanism by which probiotic strains inhibit the colonisation of pathogenic bacteria, as in *B. lactis* LKM512, which prevents the adhesion of *Clostridium perfringens* by competitive binding (Matsumoto *et al.*, 2002). The selected strains should also undergo a competitive adhesion assay in the presence of pathogenic strains, providing another criterion for probiotic potential.

2.4.3. Screening for *mapA* and *msa* genes Using PCR

L. brevis 512.3.1 and *L. oris* 5934.3.1 were screened for the presence of known genes which play a role in binding to mucin. Previous work showed that a *mapA* gene fragment could be

amplified from strains 5934.3.1 and 512.3.1 using degenerate primers (Juste-Poinapen, 2007). In contrast to this, repetition of this experiment yielded no *mapA* amplicon from these two strains, although the correct identities of these strains were confirmed by RAPD analysis (technique discussed in chapter three). In addition to the apparent absence of amplicons using the degenerate *mapA* primers, specific primers designed to the *mapA* of *L. reuteri*, strain 104R, failed to amplify any fragment from strains 5934.3.1 and 512.3.1, although it did amplify *mapA* from the *L. reuteri* ATCC 23272^T. This suggests that there may be no *mapA* gene present in either of these strains, or that the sequence of the *mapA* gene is very different from other *mapA* genes. This is in contrast to reports of high sequence homology at the protein level, the protein sequences of five different strains of *L. reuteri* sharing 98% homology (Miyoshi *et al.*, 2006).

The *mapA* gene was originally identified in *L. reuteri* 104R (Miyoshi *et al.*, 2006), and to date has been identified in only one other species, *L. plantarum* 423 (Ramiah *et al.*, 2007). It is possible that a homologue of this gene is present in strains 512.3.1 and 5934.3.1, but with dissimilar sequences in the regions where the chosen degenerate and *L. reuteri* primers bind. This possibility could be tested for using Southern hybridisation with a *mapA* probe generated from *L. reuteri*.

PCR with primers designed to the conserved regions of the *msa* gene of *L. plantarum* WCFS1 (Gross *et al.*, 2010) did not yield any amplicons from the five selected strains, suggesting that there is no gene with similar conserved sequences corresponding to the *msa* gene from *L. plantarum* WCFS1 in these strains. The incompatibility of *L. plantarum* primers with *L. oris* is not unexpected, as *L. plantarum* is more closely related to *L. brevis* than to *L. oris*, and all of these strains belong to different lactobacilli groups according to the phylogenetic analysis published by (Canchaya *et al.*, 2006).

The LbMsa primers were designed to a region of the *L. brevis* ATCC 367 genome sequence which gave maximum protein sequence alignment to the protein sequence of *msa* from *L. plantarum*, using the tBLASTn algorithm. Nevertheless, comparison of the primer sequences to sequences in the GenBank database using the primer BLAST algorithm show that the LbMsa primers are specific to *L. brevis* strains (results not shown).

While no amplicon could be obtained using primers designed to the *msa* gene of *L. brevis* ATCC 367 using annealing temperature of 50°C, amplicons of unexpected sizes were obtained when an annealing temperature of 44°C was used. It is possible that one of these amplicons represents an *msa* fragment, especially as *msa* genes are highly variable. (Gross *et al.*, 2010) showed that the *msa* genes from seven *L. plantarum* strains have seven different sequences.

Of the five selected strains screened it is most probable for strain 512.3.1 (an *L. brevis*) to harbour an *msa* gene similar enough to give an *msa* amplicon of atypical size using primers designed to a *Lactobacillus brevis* strain. Cloning and sequencing of the most abundant amplicon from strain 512.3.1 showed that this amplicon had maximum identity, at the protein level, to a portion of the *L. brevis* ATCC 367^T sequence outside of the region to which the LbMsa primer were designed. This confirms that the most abundant amplicon from strain 512.3.1 using the LbMsa primers was a non-specific PCR product. However, the inability of the PCR screen to detect an *msa* gene fragment does not mean that there is no such gene present in the selected strains.

2.4.4. Southern Hybridisation

L. brevis 512.3.1 is the selected strain which is most closely related to *L. brevis* ATCC 14869^T, the strain in which a putative homologue of *msa* was found via bioinformatic

analysis. Therefore, of the selected strains, this is the strain which is most likely to contain a homologue of *msa*. This hypothesis was explored using Southern hybridisation.

The detection of a 2.4 kb *msa*-containing fragment in *L. brevis* ATCC 14869^T using a probe generated from the same strain indicates that the Southern hybridisation was effective for sequences identical to that of the probe. This also showed that limited background signal, due to nonspecific binding of the probe, was present. There was no detectable binding of the probe to *Dra*I digested DNA from strain 512.3.1. This suggests that there is no sequence in this strain that is highly similar to the sequence of the probe. However, due to the sequence variation found among *msa* genes from different strains belonging to the same species (Gross *et al.*, 2010), it is possible that the *msa* gene of strain 512.3.1 is dissimilar enough from that of the probe to prevent binding at the stringency used in this experiment. Repeating this hybridisation using lower stringency washes may reveal the presence of such a gene.

Since the identification of the gene encoding Msa in *L. plantarum* WCFS1 in 2005 (Pretzer *et al.*, 2005), similar genes have only been identified in other *L. plantarum* strains (Gross *et al.*, 2010). The PCR primers were designed to a region of the *L. brevis* 14869^T corresponding to a putative homologue of the *L. plantarum* Msa protein. However, the functionality of this gene in *L. brevis* 14869^T has not been proved by adhesion of mannose residues. This could be further investigated by mutational analysis.

While the presence of the *mapA* and *msa* gene could not be detected using these methods, it is possible that there are functional homologues present, whose sequence is so diverse from the strains used for primer design that no genes could be detected using either PCR or southern hybridisation. These techniques are only able to detect genes with similar sequences. Another strategy which could be used is to subject proteins extracted from strains 512.3.1 and 5934.3.1 to SDS-PAGE, and probe for mucin binding proteins using horse radish peroxidase - labelled mucin. This would reveal the presence of any protein that adheres to mucin, and

allows for protein sequence analysis by MALDI-TOF if the protein can be successfully isolated. This strategy has been successfully employed recently to identify adhesins in *Lactobacillus* spp. (Wang *et al.*, 2008; Macías-Rodríguez *et al.*, 2009). Alternatively, a genomic library of these strains could be constructed, and using a suitable host, clones could be screened for increased mucin adhesion capabilities. Due to the labour intensive nature of the mucin adhesion assay, screening for a mucin binding protein using horse radish-labelled peroxidase in one strain would be more efficient than screening for function in multiple clones.

It is possible that no *mapA* or *msa* genes are present in these strains, and the ability to adhere to mucin is associated with an unrelated gene. Possible candidates identified in the literature, such as Mub, Ef-Tu and Gro-EL are discussed in chapter one. While the mechanism responsible for the ability of these strains to bind to mucin is unclear at present, this ability is a desirable one. The five selected probiotic strains, including strain P1.2 and 5934.3.1, identified as *Enterococcus faecalis* and *Lactobacillus oris* respectively, all possess *in vitro* characteristics which are advantageous in potentially probiotic strains, and belong to species which are generally regarded as safe for consumption. These five selected strains were therefore considered to be suitable candidates for inclusion in an *in vivo* feeding trial (chapter three).

CHAPTER 3

CULTURE - BASED ANALYSIS OF PROBIOTIC FEEDING TRIALS

3.0.	Summary	58
3.1.	Introduction.....	59
3.2.	Methods and Materials.....	61
3.3.	Results.....	65
3.3.1.	RAPD Optimisation	65
3.3.2.	RAPD Analysis of Gut Microbiota.....	65
3.3.3.	Probiotic Feeding Trial	69
3.3.3.1.	Ostrich Survival and Mass.....	69
3.3.3.2.	Bacterial Titres of Faecal Samples	70
3.4.	Discussion	74
3.4.1.	RAPD Optimisation.....	74
3.4.2.	RAPD Analysis of Gut Microbiota.....	74
3.4.3.	Probiotic Feeding Trial	76
3.4.3.1.	Ostrich Survival and Mass.....	76
3.4.3.2.	Bacterial Titres of Faecal Samples	78

3.0.Summary

Random amplification of polymorphic DNA (RAPD) was assessed as a method for detecting the presence of the selected strains in ostrich faecal samples. It was determined that this method was not appropriate for the analysis of a large scale trial. Feeding trials were carried out in day old ostriches. Chicks were treated with an encapsulated mixture of the five selected strains and/or the antibiotic tylosin. The survival of the chicks was improved by treatment with tylosin and chicks treated with the probiotic had an observed increase in weight gain. The culturable microbiota was assessed by aerobic and anaerobic culture on complex and selective media appropriate for the enumeration of total anaerobes, lactic acid bacteria, Clostridia, *Salmonella* spp. and *Enterobacteriaceae*. There is a clear change in the culturable gut microbiota, which coincides with a decrease in survival of chicks not receiving tylosin.

3.1.Introduction

Ostrich (*Struthio camelus*) chicks raised using intensive farming practices have a high mortality rate. Mortality is highly variable, being reported as high as 74.8% in the first three months of life (Cloete *et al.*, 2001). Some common causes of death are enteritis, colitis, fading chick syndrome, and infection of the GIT by opportunistic microorganisms (Huchzermeyer, 2002; Shivaprasad, 1993). Enteritis, an inflammation of the small intestine, has been observed to bring about more deaths in ostrich chicks than any other single cause (Huchzermeyer, 2002). Enteritis is usually produced by *Clostridium* spp. or Gram negative bacterial infections. While intensively farmed chicks have high mortality rates, those of chicks raised in the presence of their parents is lower (Kennou Sebei and Bergaoui, 2009). This may be due to many factors, but one behavioural trait of ostrich chicks raised with their parents is of particular interest. Chicks raised in the wild peck at the faeces of their parents. This pecking could provide an initial inoculum of bacteria which could aid in the development of their gut microbiota (Cooper *et al.*, 2010). Unfortunately, little is known about the microbiota of healthy ostriches, and the research which has been done is focussed on bacteria which digest fibre (Matsui *et al.*, 2010b). In previous studies, potentially probiotic bacteria were isolated from adult ostriches and characterised for their potential as probiotics (Juste-Poinapen, 2007). The bacterial strains used in this work were selected using criteria discussed in chapter one. Bacterial strains can be selected using their *in vitro* characteristics, but their probiotic activity can only be assessed by an *in vivo* trial.

During such a trial, the presence and effect of the probiotic strains on the gut microbiota can be assessed by culture dependent or culture independent methods. Culture dependent methods include enumeration by plating, random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and other methods. Culture dependent methods are often cost effective and can be less labour intensive. However, some of these

methods have poor reproducibility and do not enable the definitive identification of the bacteria without further investigation (Temmerman *et al.*, 2004). Culture dependent methods do have limitations, and should be coupled with culture independent methods to provide a more accurate view of the overall changes in the microbiota.

A combination of five selected strains was fed to ostrich chicks in order to determine the effect of the bacteria on their weight gain and survival. Some of these ostrich chicks were treated with a macrolide antibiotic called tylosin, which is common farming practice. Tylosin has been shown to be effective in preventing necrotic enteritis caused by *Clostridium perfringens* in chickens (Collier *et al.*, 2003). Macrolide antibiotics show antimicrobial activity against gram positive bacteria and few gram negative bacteria by inhibiting protein synthesis via binding to the 50S ribosome (Hamscher *et al.*, 2006).

In this study, RAPD was evaluated as a method for monitoring changes in the ostrich microbiota, and for detecting the presence of the five selected probiotic strains in faecal samples. In addition to this, the culturable bacteria were monitored by enumeration through dilution and plating. The weight gain and survival of the ostrich chicks during treatment with selected, potentially probiotic strains, both in the presence and absence of prophylactic antibiotic treatment was also measured.

3.2.Methods and Materials

Strains: Five selected strains were used: *Lactobacillus oris* 5934.3.1, *Lactobacillus brevis* 512.3.1, *Lactobacillus johnsonii* 136.2.2J, *Bifidobacterium pseudolongum* ss *globosum* 136.3.1 and *Enterococcus faecalis* P1.2. All were isolated from farmed ostriches, except for strain P1.2, which was isolated from a wild adult ostrich. Strains were cultured anaerobically as described in chapter two.

Pilot Feeding Trial: Conducted by Juste-Poinapen (2007). Briefly, two treatment groups of one hundred ostrich chicks, at three days of age, each were treated with nothing (group C, or control) or a combination of the five selected strains (group A). The probiotic strains were encapsulated in alginate feed, at similar doses used in this study. Faecal samples were collected prior to treatment (week 0) and at two weeks after treatment. The samples collected by Juste-Poinapen (2007) were used to assess the suitability of RAPDs for analysing feeding trials.

RAPD: Colonies used in RAPDs were cultured from faecal samples from ostrich chicks during the pilot feeding trial described above. Ten colonies were randomly selected from each faecal sample cultured on De Man Rogosa Sharpe (MRS) agar ((Merck), 15 g/l bacteriological agar and 0.05% cysteine hydrochloride (Sigma Aldrich)). Crude cell extracts were prepared by bead beating. Colony material was resuspended in sterile distilled H₂O containing 0.1 mm zirconia/silica beads and bead beaten using a Mini-Beadbeater-1 (Biospec Products, Oklahoma, U.S.A.) at 4800 oscillations/min for 140s. The suspension was left at 4°C for 10 min. The supernatant was removed and stored at -20°C before use as template. RAPD reactions were performed using primers OPL5 (5'-ACGAGGCAC-3') and PL1 (5'-ACGCGCCCT-3') (Tilsala-Timisjärvi and Alatossava, 1998). PCR reactions had a total volume of 25 µl, containing 0.2 mM dNTPs, 0.5 U Kapa2G Robust Taq (Kapa Biosystems, R.S.A.), 1x buffer B (at 1.5mM magnesium chloride), 0.5 µM each primer and 5 µl template.

PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems) using the following cycling conditions: an initial denaturation step at 94°C for 2 min, 40 cycles of denaturation at 94°C for 15 s, annealing at 37°C for 30 s and elongation at 72°C for 2 min, and a final elongation step at 72°C for 10 min. Amplicons were separated by electrophoresis on a 2 % agarose gel (TAE) at 5.5 V.cm⁻¹ for 90 min and visualised using ethidium bromide staining under short wavelength UV light in a Gel Doc XR (Bio-Rad).

RAPD Optimisation: The method used was as described above, varying the Taq used in the PCR reaction, and samples were bead beaten for either 90 s or 140 s. Two different Taqs were tested. Robust Taq was used in a reaction mix as described above. Kapa Readymix reactions had a total volume of 25 µl, containing 1x Kapa Readymix (Kapa Biosystems, R.S.A.), (MgCl₂ concentration of 1.5 mM), 0.5 µM each primer (OPL5 and PL1 above) and 5 µl template. Amplicons were subjected to electrophoresis as described above.

Feeding Trials: Study was carried out at Elsenburg Agricultural Centre (Stellenbosch, South Africa). Two hundred and nine ostrich chicks were obtained from two different hatcheries three days after hatching. This was designated day 0 of the trial. The 209 birds were tagged and weighed. These were randomly sorted into 12 groups with similar mass distributions, the smallest chick weighing 0.581 kg and the largest weighing 1.175 kg. Three replicate groups were assigned to each of 4 treatments (Table 3.1) in such a way that similar treatments were not situated in neighbouring pens. Groups were designated a name according to their treatments as seen in Table 3.1. Chicks had access to starter feed and water *ad libitum*. The starter feed was formulated and mixed by Elsenburg Agricultural Centre. Tylosin treatment was given via feed at a dosage of 50 mg/kg of starter feed. A combination of the five selected strains was encapsulated in alginate as described by (Juste-Poinapen, 2007). Each probiotic treated chick was fed ~10⁹ cfu probiotic bacteria (one alginate capsule) immediately after sorting and again after one week. From week two, 300mg alginate beads (~ 6x10⁹ cfu) was

Table 3.1: Treatment of groups of ostriches.

Group	Replicate	Tylosin	Probiotic	Number of Chicks
C	1	-	-	17
	2	-	-	17
	3	-	-	17
N	1	-	+	19
	2	-	+	17
	3	-	+	18
CT	1	+	-	17
	2	+	-	17
	3	+	-	17
NT	1	+	+	19
	2	+	+	17
	3	+	+	17

mixed into the feed daily. Treatment ceased after nine weeks. Birds were weighed weekly from day 0. Average weight and survival of chicks was subjected to statistical analysis using students' t-test.

Sampling: At day 0, three faecal samples were taken from each pen prior to first probiotic treatment and stored in Transport Swabs (In vitro diagnosticum, Oxoid). Thereafter samples were taken at weeks 1, 2, 4, 7, 9 and 11. A small portion of each of 5 samples were stored in transport swabs, while the bulk of the faecal matter was stored at -20°C in sterile plastic bottles immediately after transport at 4°C. Deceased birds were refrigerated and transported to a veterinarian for post-mortems within one week of death.

Culture Techniques: Faecal matter from transport swabs was resuspended in 1 ml phosphate buffered saline, pH 7.4 (PBS). Equal volumes were pooled from three samples from the same treatment group replicate. This was diluted in PBS and plated at 100 fold dilution intervals on 5 different media. Brain Heart Infusion (BHI) agar ((Bacto), 0.05% cysteine hydrochloride (Sigma Aldrich), 15g/l bacteriological agar (Merck)) was used to culture total anaerobes. De Man Rogosa Sharpe (MRS) agar ((Merck), 0.05% cysteine hydrochloride (Sigma Aldrich), 15g/l bacteriological agar (Merck)) was used for the culture of lactic acid bacteria (LAB). Tryptose Sulphite Cycloserine (TSC) agar (46g/l Perfringens agar base (Oxoid), 2 vials/l TSC

supplement (Oxoid)) was used to culture *Clostridium perfringens* and other *Clostridium* spp. Salmonella Shigella (SS) agar (Merck) was used a growth medium for *Salmonella* spp. and MacConkey agar (Merck) was used to culture lactose positive bacteria, such as *Enterobacteriaceae*. SS and MacConkey agar plates were incubated aerobically for 24 hours before being enumerated, while the other plates were incubated anaerobically for two days as described in chapter two. Black colonies on TSC agar were enumerated, as were the pink colonies on MacConkey agar and the colonies with black centres on SS agar. All colonies were counted for other growth media. Titres of bacteria were subjected to statistical analysis using students' t-test.

3.3.Results

3.3.1. RAPD Optimisation

A technique for discriminating between bacterial species cultured from faecal samples was required in order to analyse the progress of a feeding trial. Before the efficiency of this test could be examined, the RAPD PCR had to be optimised for the selected strains. This would ensure that this RAPD PCR could show which cultured colonies were the selected strains.

The RAPD PCR was optimised in terms of the Taq used and the preparation of the template prior to PCR. Figure 3.1 shows the five probiotic strains subjected to RAPD PCR using Kapa Readymix and Kapa Robust Taq respectively. The template in these reactions was subjected to bead beating for 140 s. The gel photographs clearly show that the Kapa Robust Taq has amplified more bands per lane. Figure 3.2 shows the difference in patterns obtained when bead beating for 90 s and 140 s. While there is a more subtle difference between the patterns in this image, the 140 s treatment does give a clearer pattern for some strains, especially 136.2.2J (lanes 5 and 10) and 5934.3.1 (lanes 2 and 7).

3.3.2. RAPD Analysis of Gut Microbiota

RAPD analysis was evaluated as a possible method for detecting the presence of fed strains in faecal samples. The samples used to test this method were collected from ostriches during a pilot feeding trial, conducted by (Juste-Poinapen, 2007).

Figure 3.1B shows the patterns obtained for the potentially probiotic strains used in this pilot study. Figure 3.3 is a representation of the different profiles obtained in all RAPD PCR reactions using faecal DNA as template. The patterns shown in this figure were designated profiles 1 – 12. The frequency of occurrence of these patterns can be seen in Table 3.2. There are two instances of a RAPD pattern of a faecal sample corresponding to that of one of the probiotic strains administered in the feed. These are pattern 5 (Fig 3.3, lane 5), which was

seen in bird C2 at day 0, and pattern 7 (Fig 3.3, lane 7), which appeared in bird C3 at day 0. Both patterns appear to be similar to that of strain 512.3.1 (Fig 3.1B, lane 2), which is a *L. brevis*.

The numbers of profiles per treatment group, detailed in Table 3.2, show the variation within the 10 colonies randomly chosen from each bird. Each bird had between one and four different RAPD patterns. These samples were taken from three birds out of a group of one hundred. There was less variation in the patterns obtained from the group A (probiotic fed) birds at day 0, than the control birds. In contrast to this, after two weeks, there appears to be greater variation in the patterns obtained from the probiotic fed birds than the control birds, two of which (C1 and C3) had only one profile each, or no variation.

Profile 1 (Fig 3.3, lane 1) appeared in all birds at day 0, but only in one bird (C2) at week 2.

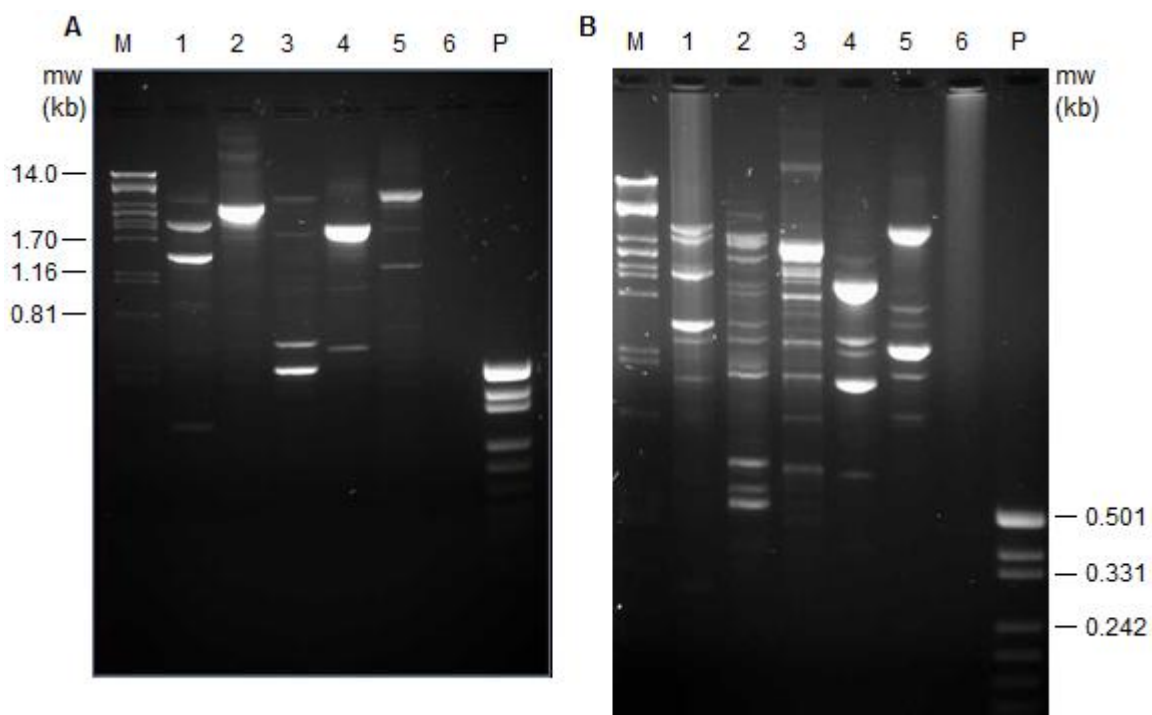


Figure 3.1: Gels showing amplicons from RAPD PCR using Kapa Readymix (A) and Kapa Robust Taq (B). M: λ DNA digested with *Pst*I. P: pUC19 digested with *Hpa*II. (A) Lane 1: 136.3.1. Lane 2: 5943.3.1. Lane 3: 512.3.1. Lane 4: P1.2. Lane 5: 136.2.2J. Lane 6: No template control. (B) Lane 1: 136.3.1. Lane 2: 512.3.1. Lane 3: 5934.3.1. Lane 4: P1.2. Lane 5: 136.2.2J. Lane 6: No template control. Mw (kb) indicates the molecular weight in kilobases of fragments in the adjacent lane.

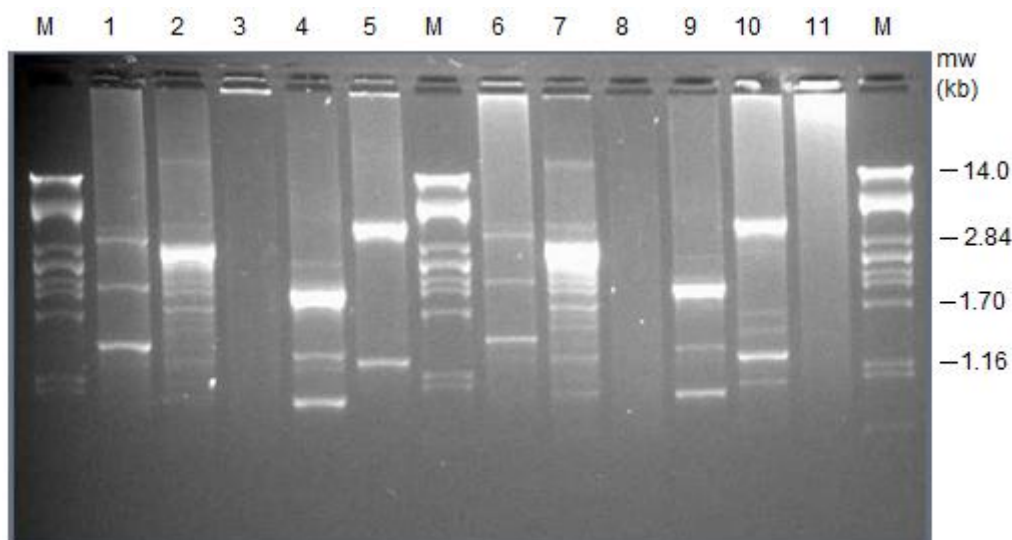


Figure 3.2: Comparison of 90 s (lanes 1-5) and 140 s (lanes 6-10) bead beating treatment for template preparation for strains 136.3.1 (lanes 1 and 6), 5934.3.1 (lanes 2 and 7), 512.3.1 (lanes 3 and 8), P1.2 (lanes 4 and 9) and 136.2.2J (lanes 5 and 10). Lane 11: no template control. M: λ DNA digested with *Pst*I. Mw (kb) indicates the molecular weight in kilobases of fragments in the adjacent lane.

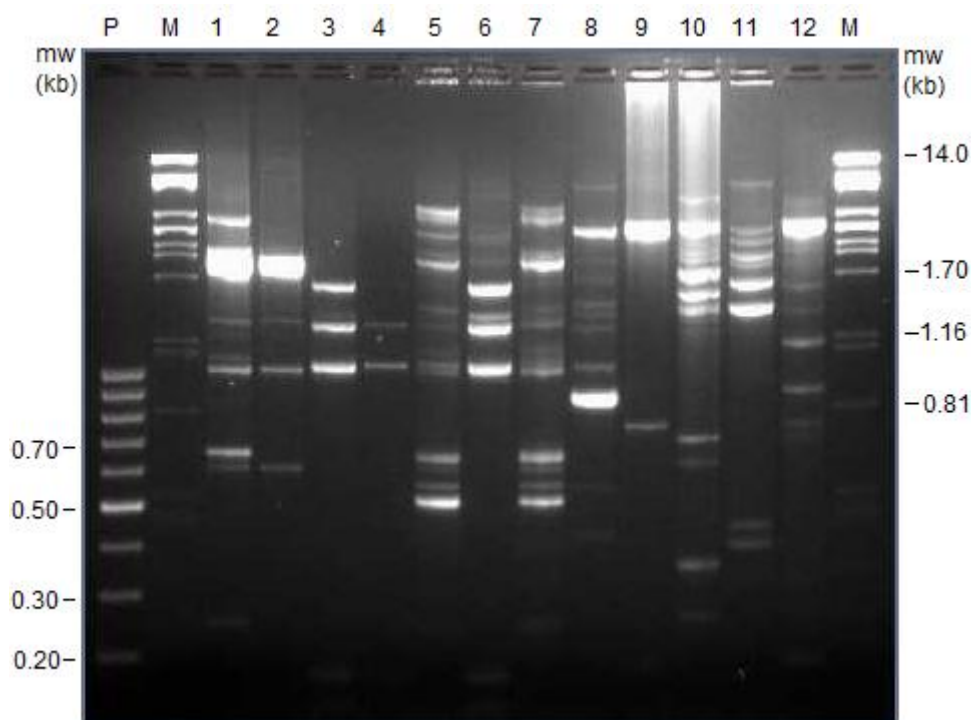


Figure 3.3: Representative patterns obtained from single colonies isolated on MRS agar from faecal samples during RAPD PCR experiments. P: GeneRuler 100bp DNA Ladder (Fermentas). M: λ DNA digested with *Pst*I. Lanes 1-12 show patterns designated profiles 1-12. Mw (kb) indicates the molecular weight in kilobases of fragments in the adjacent lane.

Table 3.2: Representative RAPD patterns from single colonies cultured from probiotic-fed group (A) and control group (C).

Time (weeks)	Treatment Group ^a	Replicate	RAPD Pattern ^b	No. Colonies	Representative Isolate	No. Patterns
0	C	1	1	5	d0 C1.1	4
			2	2	d0 C1.3	
			3	2	d0 C1.7	
			4	1	d0 C1.9	
		2	1	8		3
			3	1		
			5	1	d0 C2.9	
		3	1	7		3
			6	2	d0 C3.1	
			7	1	d0 C3.6	
	A	1	1	9		2
			2	1		
		2	1	9		2
			2	1		
		3	1	7		2
			2	3		
2	C	1	8	10	wk2 C1.6	1
		2	1	6		2
			8	4		
		3	9	9*	wk2 C3.7	1
	A	1	2	3		2
			10	7	wk2 A1.2	
		2	2	2		3
			4	1		
			10	7		
		3	2	6		3
			11	2	wk2 A3.1	
			12	2	wk2 A3.3	

a: Group A were fed probiotic for two weeks; Group C were not fed a probiotic

b: RAPD patterns (shown in Fig 3.3) from 10 isolated colonies

*No RAPD pattern could be obtained for colony C3.3

appeared in 4 birds at day 0, and in 3 birds at week 2. Profiles 3-12 occur with less frequency.

Profiles 1, 2 and 4 are the only profiles that appear at day 0 and week 2.

3.3.3. Probiotic Feeding Trial

3.3.3.1. Ostrich Survival and Mass

Over the course of the feeding trial, the survival and mass of the ostrich chicks was recorded in order to establish whether the probiotic and tylosin treatments had an affect. Figure 3.4 shows the percentage survival of the ostrich chicks over 12 weeks. At first, the survival of chicks from all groups is similar. After four weeks, the survival of groups not receiving antibiotics dropped, and after five weeks the survival of groups being fed tylosin was significantly higher than that of groups not receiving tylosin ($P \leq 0.05$). After eight weeks the percentage survival remained somewhat stable. Post mortem analysis of deceased birds indicated that most of the birds had severe colitis (results not shown).

The mean mass of each treatment group over time is shown in Figure 3.5. The mean mass of treatment group N is higher than that of the other treatment groups after week 8. However,

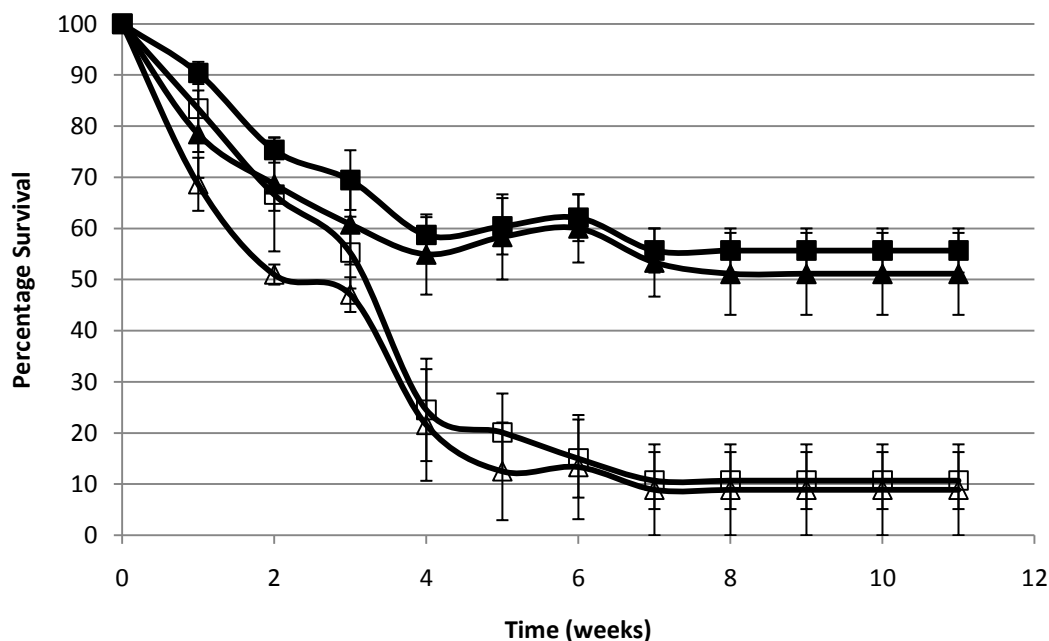


Figure 3.4: Percentage survival of ostrich chicks in treatment groups NT (■), N (□), CT (▲) and C (△). Error bars represent standard error (n=3). n represents biological replicates.

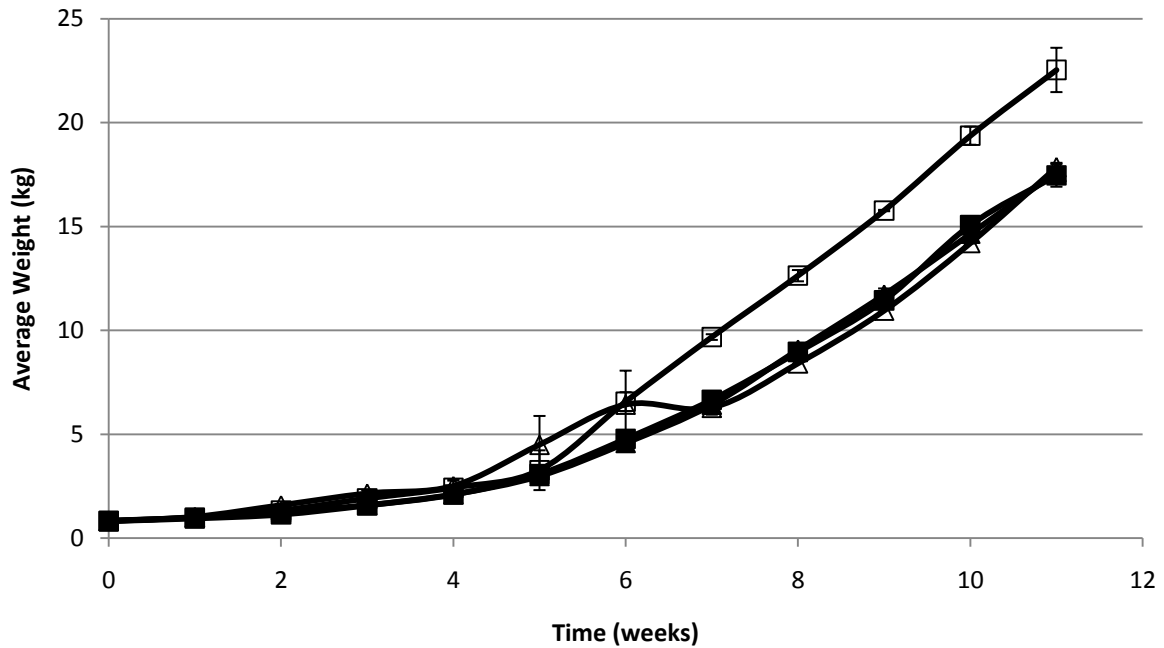


Figure 3.5: Average weights of ostrich chicks in groups NT (■), N (□), CT (▲) and C (Δ). Error bars represent standard error (n=3, except for N at 6-11 weeks, and C at 4-6 weeks, where n=2). Absence of error bars indicates n=1. n represents biological replicates.

the limited survival of group N at this time point prevented a statistical analysis, to show the statistical significance of this increase.

3.3.3.2. Bacterial Titres of Faecal Samples

Faecal samples taken from three birds from each group in the feeding trial conducted in this study were cultured on various growth media, and the bacteria were enumerated in order to assess the affect of the treatments on the culturable bacterial community.

The mean titres of the bacteria in the faecal samples from the different treatment groups on BHI agar are shown in Figure 3.6A. There is little difference in bacterial titres from faecal samples of the different treatment groups at each time point. However, the titre of group NT on BHI at 4 weeks is significantly less than the same at 2 weeks ($P<0.01$), and the corresponding titre at seven weeks is significantly greater than at four weeks ($P<0.05$).

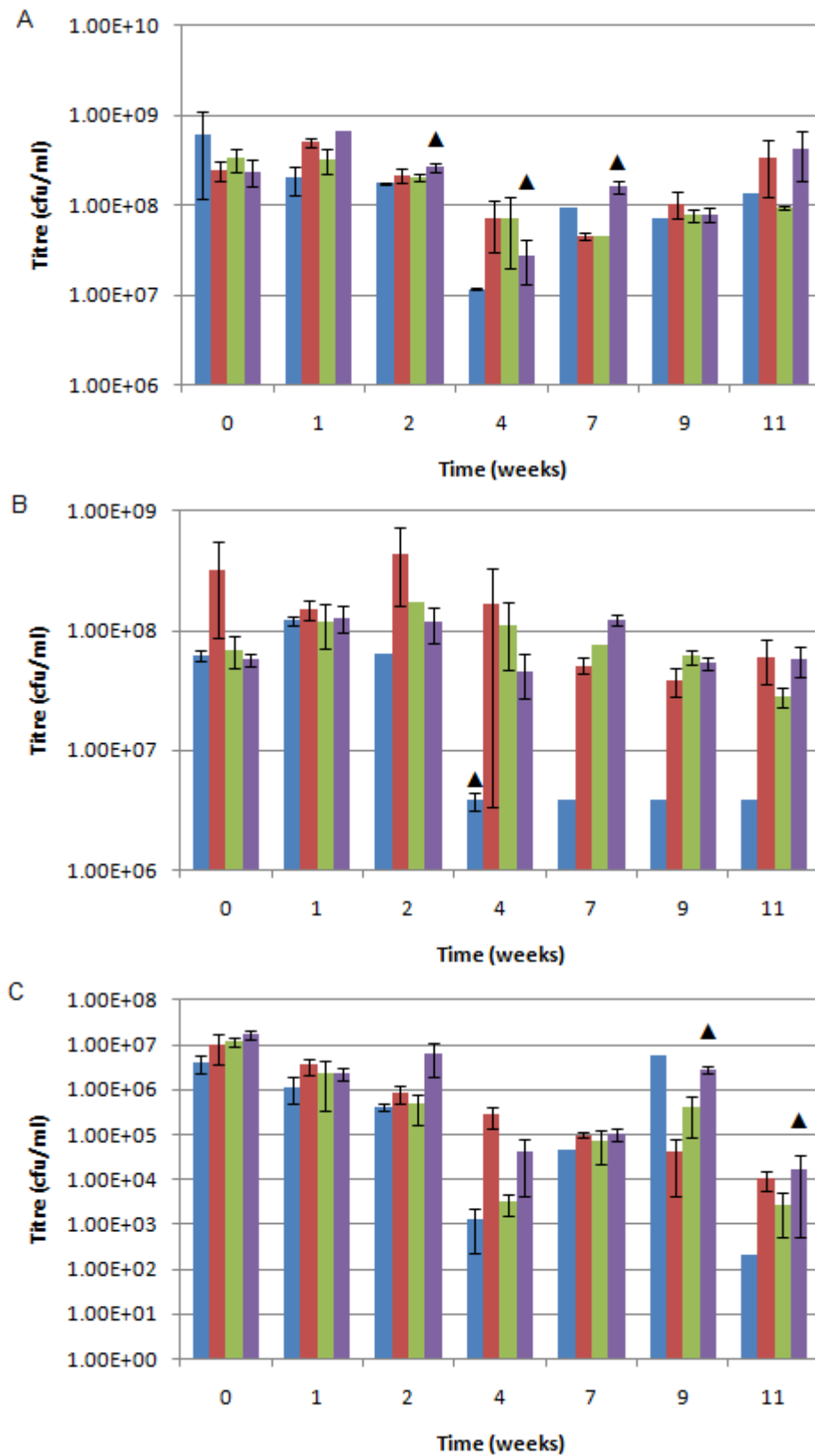


Figure 3.6: Titres of bacteria cultured anaerobically on BHI (A), MRS (B) and TSC (C) agar from treatment groups C (■), CT (■), N (■) and NT (■). Error bars represent standard error (n=3). Absence of error bars indicates that fewer than two biological samples were available. Triangles indicate points of interest referred to in the text.

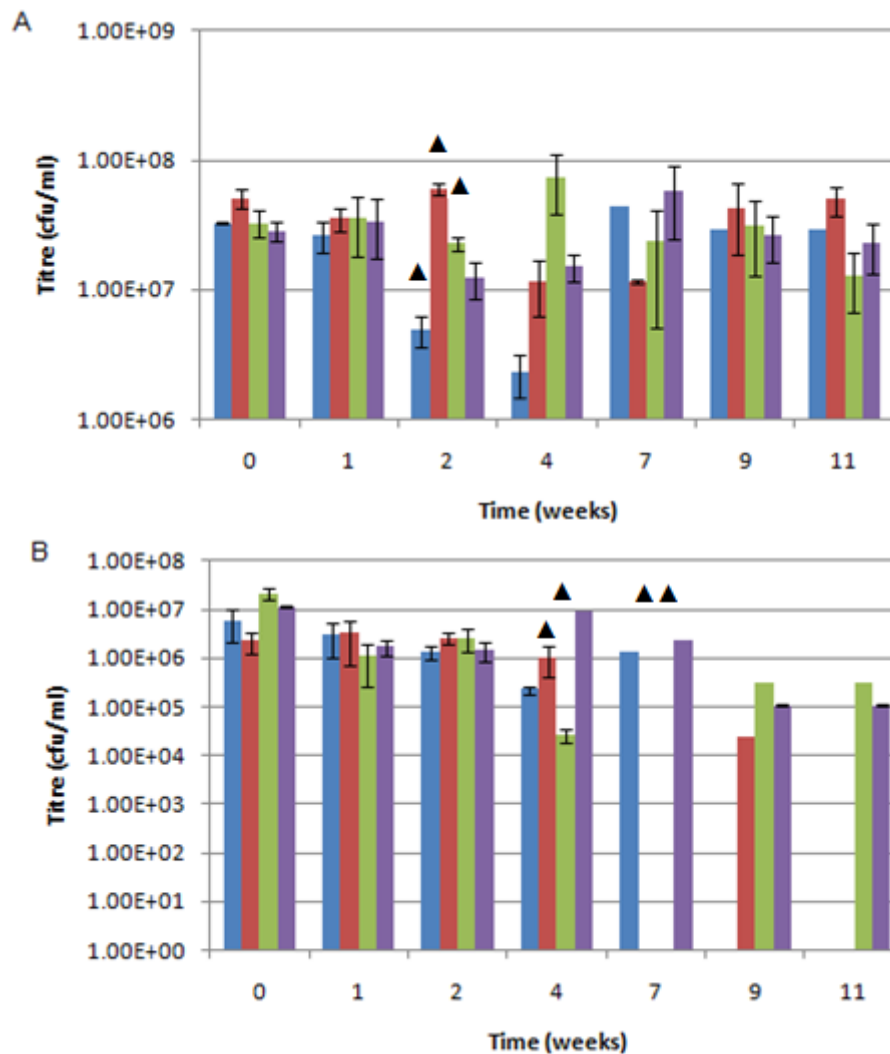


Figure 3.7: Titres of bacteria cultured aerobically on MacConkey (A) and SS (B) agar from treatment groups C (■), CT (■), N (■) and NT (■). Error bars represent standard error (n=3). Triangles indicate points of interest referred to in the text. Absence of a bar in panel B indicates a titre below 10^4 cfu/ml at weeks 7 and 9, and a titre below 10^3 cfu/ml at week 11.

Similarly, the mean titres of lactic acid bacteria for the different treatment groups cultured on MRS agar are comparable at each time point (Fig 3.6B). The bacteria cultured from group C on MRS dropped after 4 weeks, and remained very low. The statistical significance of this could not be analysed due to a lack of biological replicates, as there were only two surviving replicate groups for this treatment, and a minimum of three replicates is required for a students t-test.

Figure 3.6C shows the fluctuating titres of *Clostridium perfringens* and other *Clostridium* spp. obtained by culture on TSC agar. The mean titres at 4 weeks are less than those at 2

weeks for all treatments. However, this difference is not statistically significant ($P>0.24$). The titre of treatment group NT increases at week nine ($P<0.05$) and drops back to similar levels at week eleven ($P<0.05$).

The titres obtained by aerobic culture are shown in Figure 3.7. The mean titres of lactose positive *Enterobacteriaceae* on MacConkey agar are not significantly different in each treatment group as time progresses (Fig 3.7A). At two weeks, the titre of group C is significantly less than that of group N ($P<0.05$). Treatment group CT also has a significantly higher titre than all other treatment groups at this point. There are no significant differences between treatment groups at any other assay time.

Culture on SS agar yielded titres of *Salmonella* spp. that varied among treatment groups, with the passage of time, and even within treatment groups (Fig 3.7B). This can best be seen from week four onwards. For example, group CT and N show titres of $\sim 10^6$ cfu/ml, but these drop to zero within the next assay time, and the titres increase to similar levels once more at nine weeks. The lower limit of detection of *Salmonella* spp. for this assay is 10^4 cfu/ml at 7 and 9 weeks, and 10^3 cfu/ml at 11 weeks. The absence of a bar in Fig 3.7B at these points indicates that the titre of *Salmonella* spp. was below the limit of detection.

3.4. Discussion

3.4.1. RAPD Optimisation

The variable conditions considered during optimisation were the type of Taq used and length of bead beating during template preparation, in order to achieve complete lysis of the bacteria. Clearer RAPD patterns with more bands are produced when colonies are bead beaten for 140 s and PCR is performed using Kapa Robust Taq. These conditions were used in all remaining RAPD analyses.

The requirement for a lysis pretreatment, such as bead beating, confirms that some bacterial strains do not lyse as well as others (Vaughan *et al.*, 2000). Some of the selected strains do not lyse readily in a colony PCR, unlike other strains, such as *E. coli* DH5 α , which routinely lyses in a colony PCR without the need for any bead beating treatment. This could be due to the thick cell wall, characteristic of Gram positive bacteria. Kapa Robust Taq was developed to offer consistent colony PCR results and improve PCR efficiency in the presence of inhibitors, allowing the use of crude samples (Kapa Biosystems, 2010). The results of this optimisation show that Kapa Robust Taq does produce RAPD profiles with more bands than Kapa Taq when bead beaten colony material is used as template.

3.4.2. RAPD Analysis of Gut Microbiota

RAPD analysis of 10 individual isolates from faecal samples taken at each time point did not enable the tracking of probiotic strains administered to the birds in the treatment groups. A strain giving a similar RAPD pattern to strain 512.3.1 was present in the faecal samples in two instances, however these faecal samples were taken before probiotic feeding began and this suggests that *L. brevis* was a part of the microbiota before any probiotics were fed to the chicks. It is possible that the chicks are exposed to *L. brevis* during hatching and in their surroundings during the three days between hatching and the beginning of the feeding trial.

The identity of these two strains should be confirmed by other methods. The frequency of profiles in each bird may suggest an increase in bacterial diversity of the gut microbiota of probiotic treated birds over two weeks and a decrease in that of control birds over the same period. However, this information has been elucidated from only 10 bacterial colonies cultured from 3 birds out of a treatment group of 100 birds, and is further limited to the gut microbiota which is culturable on MRS agar. Culture independent methods, such as DGGE, may be of more use in examining the bacterial community, its diversity, and the significance of the presence of strains at certain time points.

Samples for RAPD analysis were taken from a pilot feeding trial, which had few biological replicate groups. The samples from this trial were used to determine the best methods for analysing the microbiota. The RAPD PCR does indicate some variety in the culturable microbiota. However, this method only provides limited information about the gut composition, as only the microbes able to grow on MRS agar can be detected, and the exact identities of these strains would have to be confirmed by sequencing of the 16S rRNA gene.

RAPD analysis has been successfully used to show the persistence of probiotic *Lactobacillus* spp., indicating different numbers of culturable isolates, depending on the information required from the analysis. Feeding trials in humans (Mahenthiralingam *et al.*, 2009) and pigs (De Angelis *et al.*, 2007) used 10-15 isolates to confirm the presence of the fed strains in faecal samples. However, in order to obtain meaningful information about the frequency of presence of different RAPD isolates, 91-126 isolates were required from each sample in a feeding trial for weaning piglets (Takahashi *et al.*, 2007), and it is recommended that several hundred isolates are identified in order to obtain dependable statistics (Vaughan *et al.*, 2000). In order to obtain a similar depth of detail from the pilot trial in this chapter, approximately 10 times more (in the order of 1200) strains would have to be cultured, analysed, and

sequenced to confirm their identity. This would be a costly exercise to elucidate information about only the portion of the gut microbiota which is culturable on MRS agar.

Another strategy employed to detect a probiotic using RAPD analysis is to decrease the original number of bacteria cultured, via the use of antibiotic selection. In addition to culture of faecal samples on MRS as performed in this study, faecal samples are cultured on MRS supplemented with antibiotics to which the fed probiotic is known to be resistant, such as polymixin B, chloramphenicol or streptomycin (Mahenthiralingam *et al.*, 2009; De Angelis *et al.*, 2007). Selecting for bacteria which have similar antibiotic resistance to the probiotic fed bacteria allows the screening of fewer isolates in order to detect this strain in faecal samples.

In contrast, denaturing gel electrophoresis (DGGE) can be used to analyse unculturable organisms, and can reveal the presence of numerous bacterial species in the microbiota simultaneously. The nature of DGGE means that there is no culture bias, and more meaningful data may be obtained using this method rather than selective culture and RAPD PCR (Temmerman *et al.*, 2004). Increasing the population coverage would increase the reliability of this data, however, its qualitative nature makes it less useful than a quantifiable method that can be statistically analysed. It was decided that future feeding trials would be studied using a combination of analysis of bacteria cultured on specific media, and the culture independent method, DGGE.

3.4.3. Probiotic Feeding Trial

3.4.3.1. Ostrich Survival and Mass

Treatment group N showed a larger weight gain than any other treatment group. At this point there were only two remaining biological replicates, preventing a statistical analysis to show the significance of this result. Treatment with both the probiotic and antibiotic did not seem to result in the same weight gain, suggesting that this effect may be due to the action of the

selected strains which are sensitive to the action of tylosin. Treatment with tylosin did not seem to increase the weight gain of the birds in the first 11 weeks of life. However, the period during which maximum daily weight gain occurs has been described as being between 92 and 175 days of age (Cooper and Mahrose, 2004). This falls well outside of the period studied, which was between 3 and 80 days. Therefore, the absence of a larger weight gain at this age may not be representative of a larger weight gain later in life. As this was a pilot study, future studies should continue monitoring the weight gain at least until 175 days, in order to determine long term weight gain effects.

Ostrich chicks which were fed tylosin had a significantly higher percentage survival after 5 weeks ($P < 0.05$). The largest drop in survival occurred within the first 4 weeks. This agrees with the findings of (Cloete *et al.*, 2001), who reported that 60 % of ostrich chicks lost in the first 90 days of life died in the first 28 days.

This suggests that ostrich chicks should receive this antibiotic during future feeding trials, even if it is in conjunction with a probiotic supplement. While treatment with tylosin significantly increased the survival of the chicks, treatment with a combination of tylosin and the probiotic appears to have negated the effect of weight gain, which resulted from treatment with the probiotic alone. Since probiotic treatment did not increase the survival of the chicks, and the chicks were not monitored during the ages when the maximum weight gain occurs, it may be useful to repeat these trials, treating with tylosin until the age of 4 weeks to prevent a high mortality rate, and beginning probiotic treatment after this. Such a protocol would show whether probiotic treatment will result in larger weight gain after 4 weeks of age, after the survival of the chicks has stabilised.

The presence of tylosin in conjunction with a probiotic supplement may have some effect on the function of the probiotic. The ability to survive, or even grow, in the presence of tylosin

may be a favourable trait when considering the selection of future strains as potential probiotics.

3.4.3.2. Bacterial Titres of Faecal Samples

BHI agar was used to enumerate the total anaerobic community, while the bacteria cultured on MRS represent the lactic acid bacteria (LAB). *Clostridium perfringens* and other *Clostridium* spp. produce black colonies on TSC agar (Hauschild *et al.*, 1974). MacConkey agar was used to select for lactose positive *Enterobacteriaceae* and most *Salmonella* species produce straw coloured colonies with black centres on SS agar (Prescott *et al.*, 2005). The presumptive tests were considered to be adequate in this case, as the purpose of these selective media was to enumerate the microbiota of a larger number of samples and not to identify strains conclusively.

There is clearly a change in the culturable gut microbiota after 4 weeks, as titres for some individual groups showed a marked decrease after four weeks. These were group NT cultured on BHI, group C on MRS and all groups cultured on TSC. These drops in titre occurred at the same time point as the decrease in survival of chicks which were not receiving tylosin treatment. The decrease in titre of anaerobically assayed bacteria at this point may be associated with the decline in survival at week four, or may reflect lower titres of anaerobic bacteria in the surviving ostrich chicks.

Enteritis (inflammation of the small intestine) has been recorded as the cause of most premature ostrich deaths in intensive farming settings (Huchzermeyer, 2002). This is attributed to infection by *Clostridium* spp. and Gram negative bacteria. The cause of death of many of the chicks in this trial was identified as colitis (inflammation of the colon) (results not shown). Culturing the intestinal contents of deceased birds may be useful in identifying bacteria which have contributed to the cause of death in future trials. This information could

also be used to investigate interactions between the pathogenic bacterial strains and the probiotic strains.

The decrease in survival at week four was accompanied by a drop in titre of *Clostridium* spp. in the surviving chicks which received neither probiotic nor antibiotic treatment. The titres of *Clostridium* spp. remained low during the remainder of the trial. However, there is not sufficient data to infer a positive or negative effect of the culturable bacterial community of the live birds on their survival at this point. Similar probiotic feeding trials carried out in broiler chickens showed that titres of *Clostridium* spp. decreased in over time, with or without probiotic feeding (Gérard *et al.*, 2008) and a decrease in the presence of *Clostridium* spp. has also been found when chickens have been treated with probiotic strains (Wise and Siragusa, 2007; Gérard *et al.*, 2008). The birds which died during week four may have had a different bacterial profile immediately before their deaths, and the drop in titres, measured in the live birds, (including that of *Clostridium* spp.) may have contributed to their survival.

Culture based methods provide important information about the culturable gut microbiota. However, it has been estimated that only 15 – 58 % of the total microbiota is culturable, using enumeration by dilution and plating on selective media (Vaughan *et al.*, 2000). Therefore, it is best that results obtained by culture-based methods are supported by data from culture independent methods.

Feeding trials on broiler chickens used to evaluate the efficacy of a probiotic mix containing lactobacilli, bifidobacteria and enterococci used similar methods to analyse the bacterial community (Mountzouris *et al.*, 2007). These authors did not find any significant difference in the number of any of the cultured bacteria. However, they did find an increase in the feed conversion ratio of the broiler chickens. It is possible that the ostrich chicks showed an increased efficiency in feed conversion or growth during their optimal growth ages even though no difference could be detected in the culturable microbiota. In future *in vivo* trials, it

is recommended that the ostrich chicks be treated with probiotic strains in conjunction with antibiotic treatment until they have passed the age of optimal growth. This should allow any effect of the probiotic on growth of the chicks to become apparent. Alternatively, the probiotic treatment could be delayed until the fourth week of age, when the surviving chicks begin optimal growth. Future trials should also include culture of intestinal contents as part of the post mortem procedure in order to identify the bacteria causing enterocolitis.

CHAPTER 4

DGGE ANALYSIS OF PROBIOTIC FEEDING TRIAL

4.0.	Summary	82
4.1.	Introduction.....	83
4.2.	Methods and Materials.....	86
4.3.	Results.....	89
4.3.1.	Optimising DGGE Protocol.....	89
4.3.2.	DGGE Analysis of All Treatment Groups over Time	92
4.3.3.	DGGE Analysis of Similar Treatments at Different Times.....	97
4.4.	Discussion	104
4.4.1.	Optimising DGGE Protocol.....	104
4.4.2.	DGGE Analysis of All Treatment Groups over Time	105
4.4.3.	DGGE Analysis of Similar Treatments at Different Times.....	109

4.0. Summary

The feeding trials were analysed using denaturing gradient gel electrophoresis (DGGE). Groups were compared across treatments at each time point, and across time points in antibiotic treated and untreated groups. The similarity of profiles was assessed using dendrograms constructed using the Dice coefficient, and the diversity of samples was shown using the Shannon Wiener Index. The diversity and similarity of microbial profiles were shown to be significantly different as the bird ages. Selected bands which contributed to these differences were sequenced. This indicated that *Clostridium populeti*, *Clostridium symbiosum* and *Clostridium hathewayi* were present in day 0 samples, but were lost by week 9.

4.1. Introduction

While the culture dependent methods of analysis used in chapter three have their advantages, it is important to analyse the feeding trials using culture independent methods as well. These methods allow a view of the unculturable and culturable microorganisms in a sample, and should be more effective in tracking the presence of the probiotic strains in the faecal samples. Molecular methods can also be used to describe trends in the microbiota with more quantifiable data in a less labour intensive manner (Temmerman *et al.*, 2004).

The culture independent method chosen for use in this study employs denaturing gradient gel electrophoresis (DGGE). This method entails the amplification of the V3 region of the 16S rRNA gene using universal PCR primers. A subsequent PCR reaction is used to add a 40 bp GC clamp to each of these amplicons. When these amplicons are subjected to electrophoresis through a gel containing a denaturant formamide/urea gradient, the amplicons denature as the

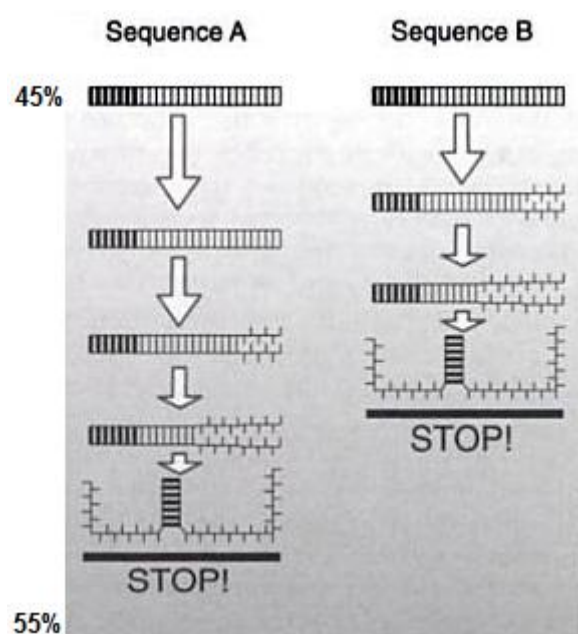


Figure 4.1: Molecular separation during DGGE. Smaller arrows indicate decreased migration speed. Migration of fragments is slowed by increasing concentrations of denaturing solution until it stops during electrophoresis. Free nucleotides, represented by small hooks, can entangle in the acrylamide matrix, causing retardation of migration. Adapted from (Felske and Osborn, 2005).

gradient increases, until the fragment reaches a point where it is wholly denatured, with the exception of the GC clamp. The fragment is impeded by the gel matrix and stops migrating. In this way, each amplicon moves to a point on the denaturing gradient which corresponds to the individual melting point of the fragment.

Visualisation using ethidium bromide is used to show a banding pattern. This banding pattern represents a part of the microbial community, where one band is generally equivalent to one species. This can in turn be subjected to statistical analysis, in order to provide some description of the diversity of each sample subjected to DGGE (Norin *et al.*, 2009).

The Shannon Wiener Index of Diversity (H') is a method of expressing diversity of a sample, taking into account both the total number of bands and the intensity of each band. This is calculated using band intensity information. The formula is:

$$H' = - \sum_{i=1}^S p_i \ln p_i$$

where p_i is the ratio of intensity of a band to the total intensity of all bands within a lane and S is the total number of bands within a lane (Shannon and Weaver, 1971). An increase in the intensity and number of bands in a lane results in an increased value for H' , and indicates an increase in diversity. Although the Shannon Wiener Index was originally developed to describe the diversity of animal populations, it has been shown to be appropriate for use in describing the diversity of microbial populations (Eichner *et al.*, 1999). However, as a result of the way in which H' is calculated, it is possible for two lanes to have equal diversity, but different banding patterns (Gafan *et al.*, 2005). In order to prevent this possibility from skewing the data, another analysis of the data should be used in conjunction with the Shannon Wiener Index of Diversity.

The Dice coefficient is an expression of the similarity of lanes in a DGGE gel to one another. This is in contrast to the measure of diversity by the Shannon Wiener Index. The dendrograms generated by Quantity One using the unweighted pair group method with arithmetic averages (UPGAMA) algorithm and the Dice coefficient only takes the number of similar bands in each lane into account, and not their relative intensities (van Rijsbergen, 1979). Using both descriptions of relatedness will allow a graphical representation of the similarity, as well as a representation of diversity, which takes the intensity of each band, or abundance of each species, into account.

One of the aims of this chapter is to establish if either probiotic treatment or age affect the diversity of the microbiota. The second aim is to determine if the probiotics used to treat the ostrich chicks are detectable in their faeces. This would support the hypothesis that these bacterial strains are surviving passage through the gastrointestinal tract with enough success that their nucleic material is intact in the faeces.

4.2. Methods and Materials

Faecal DNA Extraction: Faecal samples collected during the feeding trial as described in chapter three and stored at -20°C were partially thawed. Two hundred milligrams of faecal material was used as starting material for the ZR Fecal DNA Kit (Zymo Research, C.A., U.S.A.). Equal amounts of faecal material from 5 individual samples were combined in one extraction to represent one replicate of one treatment group as suggested by Zhou *et al.* (2007). Quality and concentration of genomic DNA was assessed as described in chapter two.

Polymerase Chain Reactions: Genomic DNA from faecal samples or pure cultures was subjected to two sequential PCRs. The first, named Round I for the purposes of this study, had a total volume of 25 µl, containing 0.5 U Kapa Taq (Kapa Biosystems, South Africa), 0.2 mM dNTPs, 0.5 µM Uni-341F, 0.5 µM Uni-515R (Table 4.1), 1x buffer A (containing 1.5 mM MgCl₂) and template, either 200 ng genomic DNA or 100 ng faecal DNA. Genomic DNA was prepared as described in chapter two. If faecal DNA was used as template, 0.6 mg/ml BSA (Fermentas Life Sciences) was included in the reactions. PCR was carried out in a GeneAmp PCR System 9700 (Applied Biosystems), using the following cycling conditions: an initial denaturation step at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 40 s, and a final elongation step at 72°C for 7 min. Amplicons from Round I were separated by electrophoresis on a 2% agarose gel (TAE) at 5.5 V.cm⁻¹ for 90 min and visualised using ethidium bromide under short wavelength UV light using a Gel Doc XR (Biorad). Good quality amplicons were used

Table 4.1: Primer sequences for polymerase chain reactions.

Name	Sequence	Reference
Uni-341F	5'-CCT ACG GGA GGC AGC AG-3'	Muyzer <i>et al.</i> , 1993
Uni-515R	5'-ATC GTA TTA CCG CGG CTG CTG GCA-3'	Lane, 1991
Uni-341F-GC	5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3'	Muyzer <i>et al.</i> , 1993

as template for a second PCR, named Round II for the purposes of this study. This PCR was used to increase the yield of the PCR before DGGE was performed. These reactions had a total volume of 50 µl, containing 1 U Kapa Taq (Kapa Biosystems, South Africa), 0.2 mM dNTPs, 0.5 µM Uni-341F-GC, 0.5 µM Uni-515R (Table 4.1), 1x buffer A (containing 1.5 mM MgCl₂) and 1 µl template (Round I PCR). If the original template was faecal DNA, then 0.4 mg/ml BSA (Fermentas Life Sciences) was included in each reaction. PCR was carried out using the same thermocycler and cycling conditions as for Round I. Amplicon quality was assessed as described above.

Optimising PCR: Reactions were carried out using either Kapa Taq (as above) or Kapa Readymix (Kapa Biosystems, South Africa). The first round reactions using this Readymix had a total volume of 25 µl, containing 1x Kapa Readymix (Kapa Biosystems, R.S.A.), 0.5 µM 341F, 0.5 µM 515R and template, 100 ng of either genomic DNA or faecal DNA. Readymix second round reactions had a total volume of 50 µl, containing 1x Kapa Readymix (Kapa Biosystems, R.S.A.), 0.5 µM 341F-GC, 0.5 µM 515R and template in the form of 1 µl of a first round PCR. All other samples treatments were as described above.

Denaturing Gradient Gel Electrophoresis: DGGE was performed using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, Mississauga, Ontario, Canada) as recommended by the manufacturer, with optimised values as follows. A denaturing gradient gel of 45 – 55% was cast. Reference strain ladder was prepared by combining 4 µl PCR product from each strain and an equal volume of tracking dye. Amplicons from faecal samples were prepared by combining 10 µl PCR product with an equal volume of tracking dye. Gels were electrophoresed at first for 10 min at 180 V, and then for 1100 V.h, typically over 16 – 18 hours. Gels were stained in 1 µg/ml ethidium bromide in TAE buffer for 30 minutes, and destained in TAE buffer for 2 minutes. Destained gels were visualised under short wavelength UV light in a GelDoc XR (Bio-Rad, Mississauga, Ontario, Canada).

Gel Analysis: Quantity One version 4.5.2 was used to correct background of photographs and detect and match bands. This analysis was used to construct dendrograms of band relatedness using the UPGAMA algorithm and Dice's coefficient. Ratios of band intensities, obtained from Quantity One, were used to calculate the Shannon Wiener Diversity Index (H') of each lane. H' values were subjected to statistical analysis using students' t-test.

Sequencing of DGGE bands: After visualisation as described above, gels were visualised using long wavelength UV light, bands were excised using a scalpel and were stored in 20 μ l sterile distilled water at 4°C overnight. From this, 2 μ l was used as template in a first round PCR as described above. Size of amplicon was confirmed by gel electrophoresis as before. Amplicons were ligated into pTZ57R/T as per manufacturer's instructions (InsTAclone PCR cloning kit, Fermentas Life Sciences). Plasmids were transformed into RbCl competent *E. coli* DH5 α (Armitage *et al.*, 1988). Plasmids were extracted from transformants (Ish-Horowicz and Burke, 1981) before undergoing PCR using M13 primers in order to screen for insert, as described in chapter two. Plasmids of appropriate transformants were extracted using a Biospin PCR Purification kit (Bioflux, Japan) as per manufacturer's instructions. Sequencing reactions were performed by MacroGen Inc (Korea).

4.3. Results

4.3.1. Optimising DGGE Protocol

The PCR protocol employed to amplify fragments of 16S rRNA genes from reference strains and faecal samples was optimised to ensure that good quality amplicons were used for DGGE analysis. In order to optimise these reactions, different Taq preparations were tested. Figure 4.2 shows that the amplicons from the Round I PCR do not differ in intensity or size when the two different Taq preparations are used. The small fragment seen in the no template control lanes was used as template in a Round II PCR to ensure that these were primer dimers. The empty lanes in Figure 4.3 confirm this. Figure 4.3 shows that the amplicons produced by Kapa Readymix are less intense than those produced by Kapa Taq.

These amplicons were mixed in equal amounts and subjected to DGGE (Fig 4.4). Strain 512.3.1 shows a more intense band when subjected to PCR with Kapa Taq than with Kapa Readymix. This figure shows the position of each strain within the mixed reference strain marker, and that the denaturing gradient of 45 – 55% separates these strains of interest. It

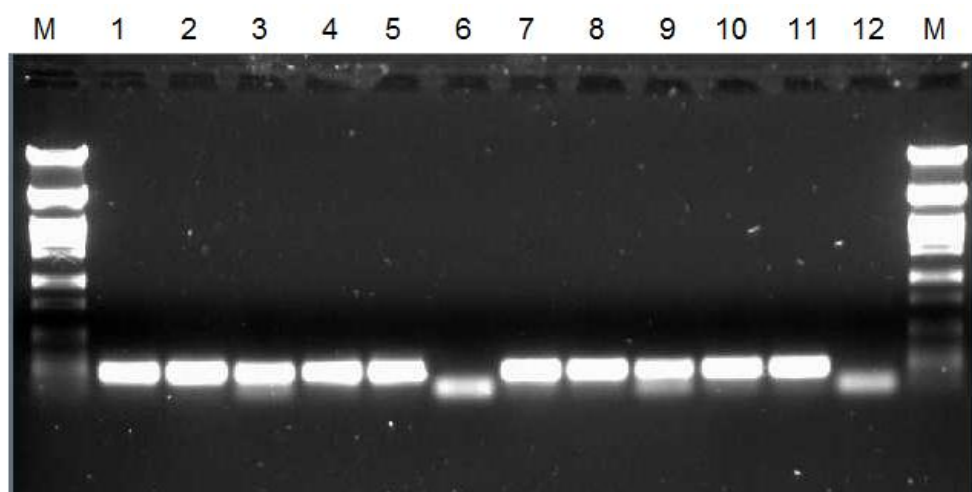


Figure 4.2: Amplicons of PCR using primers Uni-341F and Uni-515R, Kapa Taq (lanes 1-6) or Kapa Readymix (lanes 7-12) and genomic DNA from strains 512.3.1 (lanes 1 and 7), 136.2.2J (lanes 2 and 8), 136.3.1 (lanes 3 and 9) P1.2 (lanes 4 and 10) and 5934.3.1 (lanes 5 and 11). Lanes 6 and 12 represent the no template controls. M represents a molecular weight marker of *Pst*I digested λ DNA.

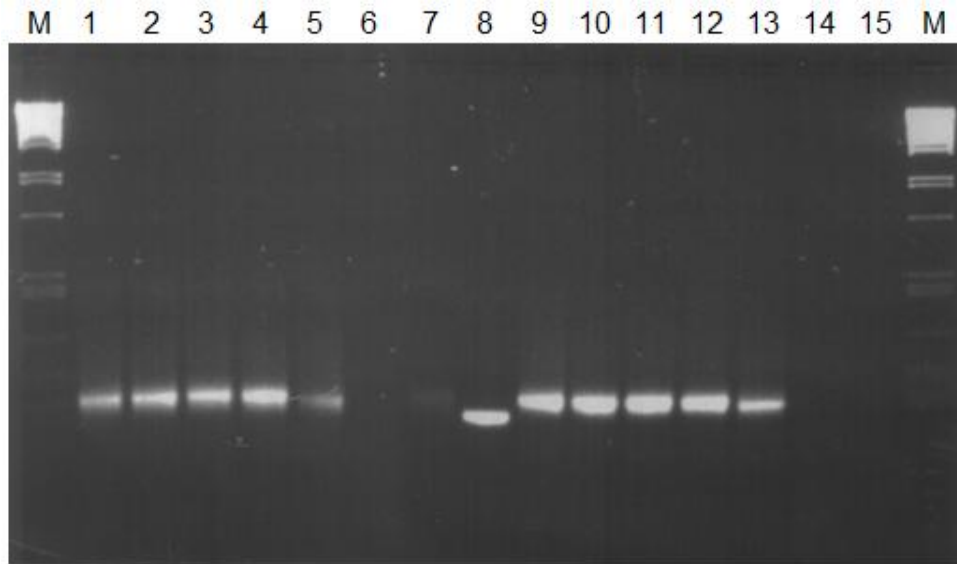


Figure 4.3: Amplicons of PCR using primers Uni-341F-GC and Uni-515R, products of the Round I PCR as template, Kapa Taq (lanes 1-7) and Kapa Readymix (lanes 9-15) for strains P1.2 (lanes 1 and 9), 512.3.1 (lanes 2 and 10), 5934.3.1 (lanes 3 and 11) 136.2.2J (lanes 4 and 12) and 136.3.1 (lanes 5 and 13). Lanes 6 and 14 represent the no template controls. Lanes 7 and 15 represent reactions where Round I no template controls were used as template. Lane 8 contains 512.3.1 amplicon from Round I PCR, to be used as a size marker. M represents a molecular weight marker of *Pst*I digested λ DNA.

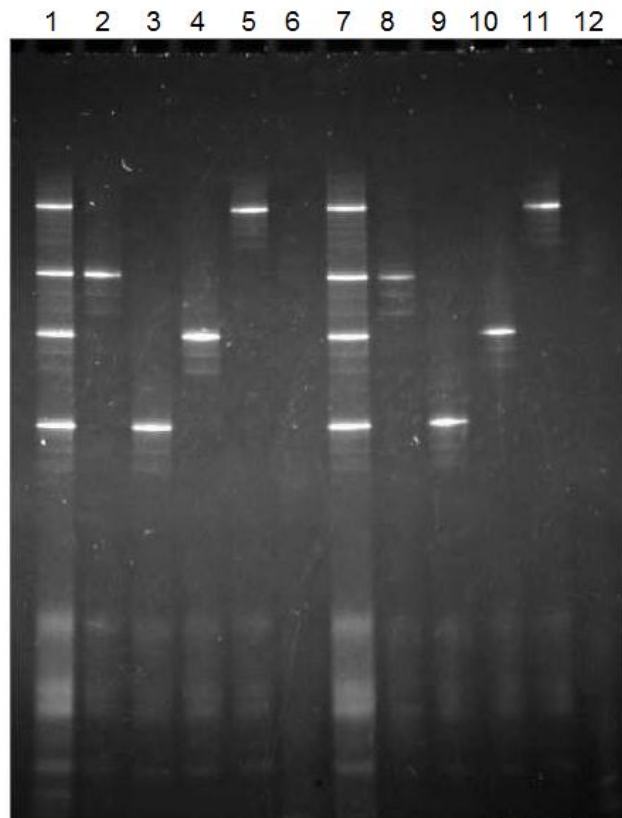


Figure 4.4: DGGE showing position and intensity of bands within reference strain ladder, when amplified using Kapa Taq (lanes 1 – 6) and Kapa Readymix (lanes 7 – 12) for strains 512.3.1 (lanes 2 and 8), 5934.3.1 (lanes 3 and 9), P1.2 (lanes 4 and 10), 136.2.2J (lanes 5 and 11) and 136.3.1 (lanes 6 and 12). Lanes 1 and 7 show the mixed reference strain ladders.

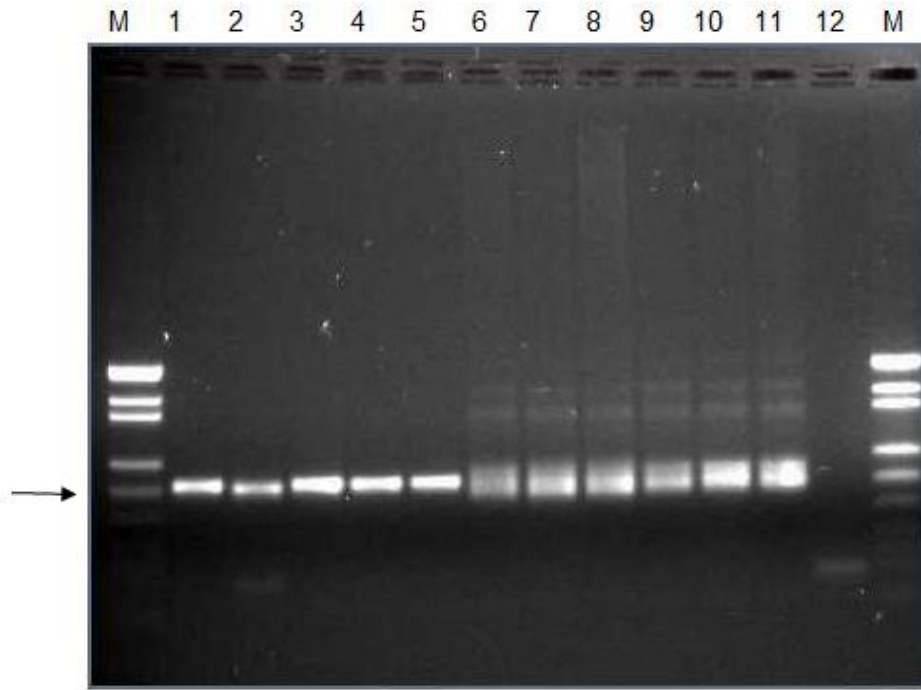


Figure 4.5: Amplicons from PCR using primers Uni-341F and Uni-515R for single strain and faecal genomic DNA. Lane 1: 512.3.1, lane 2: 136.3.1, lane 3: 5934.3.1, lane 4: P1.2, lane 5: 136.2.2J, lanes 6-11: genomic DNA extracted from different samples of ostrich faecal material. Lane 12: no template control. M represents a molecular size marker of *Hpa*II digested pUC19. Arrow indicates 190 bp fragment of *Hpa*II digested pUC19.

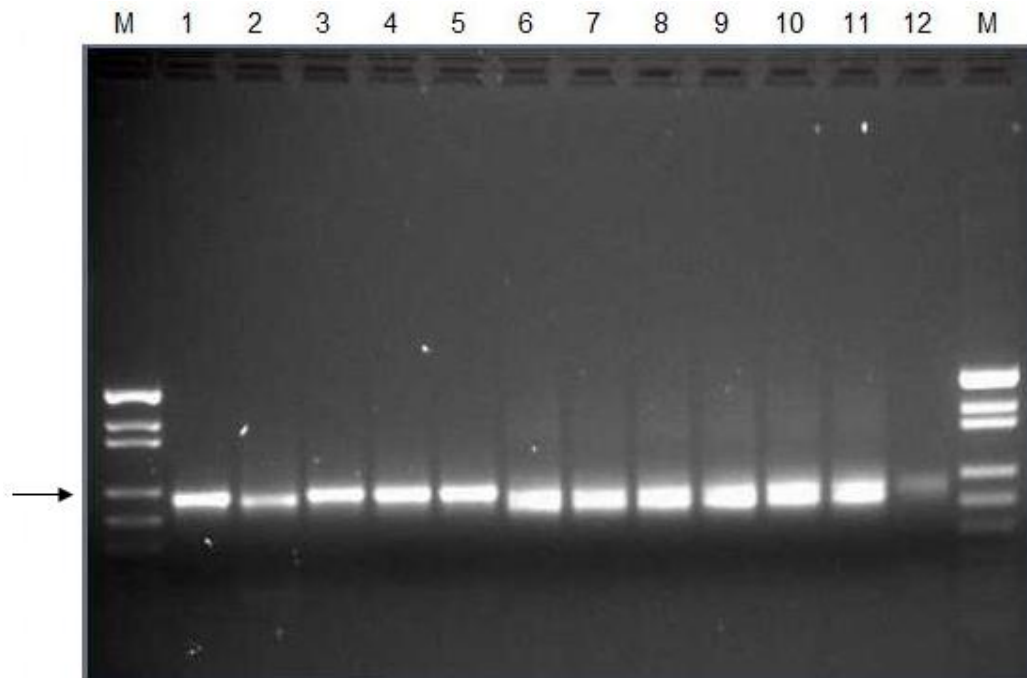


Figure 4.6: Amplicons from PCR using primers Uni-341F-GC and Uni-515R for single strain and faecal genomic DNA. Lane 1: 512.3.1, lane 2: 136.3.1, lane 3: 5934.3.1, lane 4: P1.2, lane 5: 136.2.2J, lanes 6-11: genomic DNA extracted from various ostrich faecal samples, lane 12: no template control. M represents a molecular size marker of *Hpa*II digested pUC19. Arrow indicates 242 bp fragment of *Hpa*II digested pUC19.

should be noted that while strain 136.3.1, a *Bifidobacterium pseudolongum subsp. globosum*, shows amplicons after both PCR reactions (Figures 4.2 and 4.3), no band is seen for this strain on the 45-55% DGGE gel (Figure 4.4). For clarity, the order of strains within the reference strain marker is, top to bottom, 136.2.2J (*L. johnsonii*), 512.3.1 (*L. brevis*), P1.2 (*E. faecalis*) and 5934.3.1 (*L. oris*).

Figure 4.5 shows the first round amplicons from single strain template and faecal DNA, which contains genomic DNA from many strains. A single fragment was obtained where single strain genomic DNA was used as template, although there is some nonspecific product where the template was faecal DNA. The amplicons from the second round PCR appear in Figure 4.6. The amplicons from faecal template reactions have less nonspecific product associated with them than in Figure 4.5. The fragments in Figures 4.5 and 4.6 are consistent with their expected sizes of 192 bp and 232 bp respectively.

4.3.2. DGGE Analysis of All Treatment Groups over Time

The feeding trial carried out in chapter three was analysed using DGGE analysis. During this trial, ostrich chicks were treated with 5 selected probiotic strains, an antibiotic called tylosin, a combination of both, or neither. These treatments were given via the feed to groups of ostriches in triplicate. Faecal samples were collected from five random ostriches in each group at various intervals. These five samples were combined, DNA was extracted from them, and they were subjected to PCR amplification and DGGE analysis using the conditions optimised above. DGGE analysis of treatment groups was undertaken at three time intervals, day 0 of the trial and at weeks 4 and 9 after the trial commenced. A typical DGGE gel before analysis for dendrograms and Shannon Wiener Diversity Indices is shown in Figure 4.7.

Figure 4.8B shows the similarity of the treatment groups at day 0 as calculated using the image shown in Figure 4.8A. There is no clustering according to treatment. The Quantity One

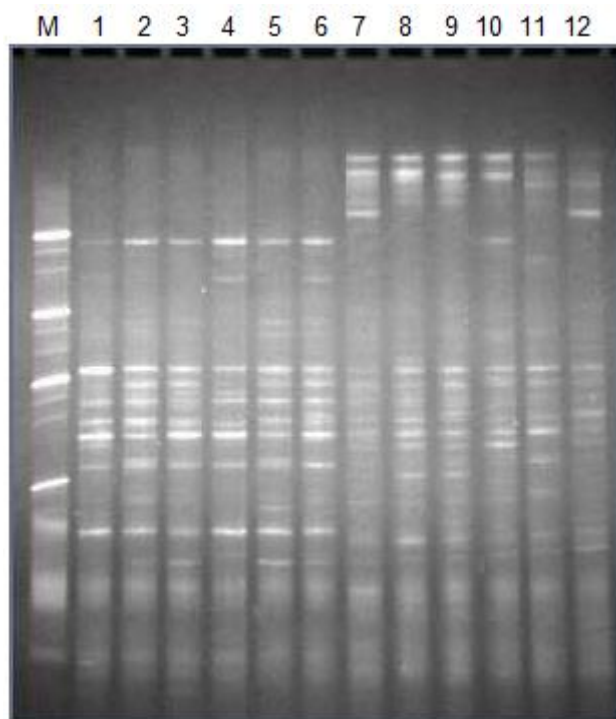


Figure 4.7: A typical DGGE gel before analysis using Quantity One. This gel shows samples from tylosin treated groups at day 0 (lanes 1-6) and week 4 (lanes 7-12). Lane 1: NT1, lane: NT2, lane 3: NT3, lane 4: CT1, lane 5: CT2, lane 6: CT3, lane 7: NT1, lane 8: NT2, lane 9: NT3, lane 10: CT1, lane 11: CT2, lane 12: CT3. M represents the reference strain marker, identities as shown in Figure 4.4. The analysed gel is shown in Figure 4.11.

analysis showed that bands in a similar position to strains 512.3.1 and P1.2 appear in the gel. Bands at a similar position to that of strain 512.3.1 appear in treatments C and CT, while bands similar to strain P1.2 are seen in all treatments. The diversity of bands shown in Figure 4.8A was further analysed using the Shannon Wiener Diversity Indices (H') shown in Table 4.2. The students' t-test showed that there was no significant difference in the value of H' across treatments ($P > 0.05$).

Similarly, there is no significant difference between the H' values calculated from the treatment groups at week 4 (Table 4.3). In contrast to this, the similarity of bacterial species within treatment groups expressed as a dendrogram (Figure 4.9) shows that four groups treated with tylosin cluster together with one non-tylosin treated group (groups NT1, CT3, NT3, NT2 and N2). The clustering of groups not treated with tylosin is less clear. Four of the

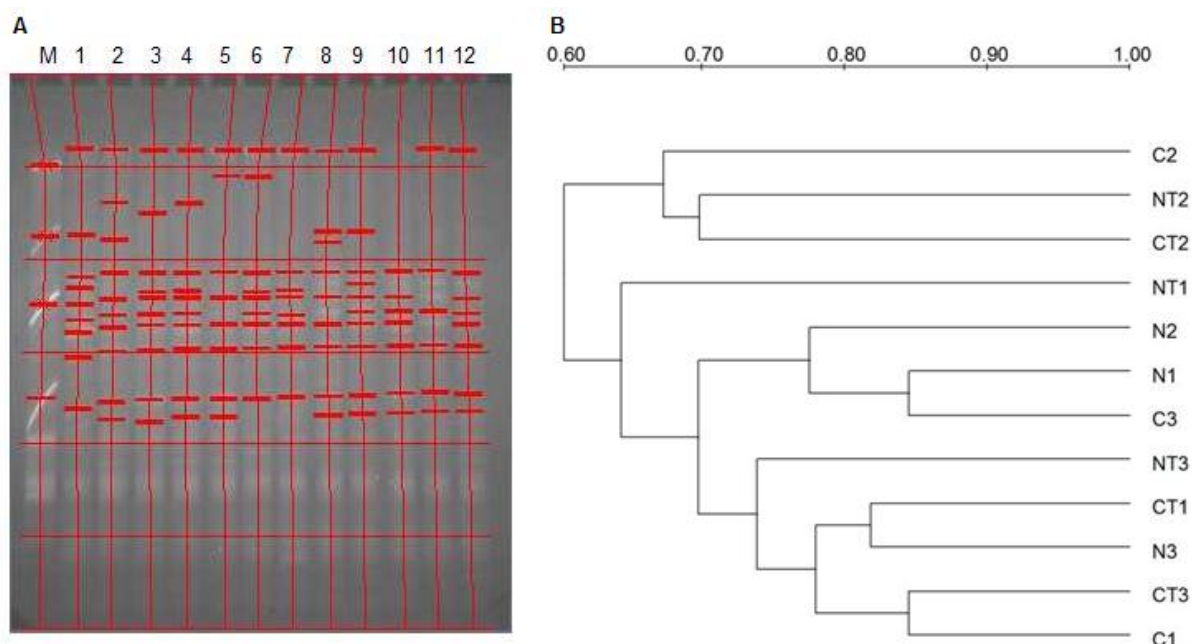


Figure 4.8: DGGE results for all groups at day 0. (A) DGGE with overlay. Red overlay indicates bands recognised and matched using Quantity One software. Lane 1: C1, Lane 2: C2, Lane 3: C3, Lane 4: N1, Lane 5: N2, Lane 6: N3, Lane 7: CT1, Lane 8: CT2, Lane 9: CT3, Lane 10: NT1, Lane 11: NT2, Lane 12: NT3. M represents reference strain marker, identities as shown in Figure 4.4. (B) Dendrogram showing relatedness of bands. Scale bar represents relative distance as Dice coefficients.

Table 4.2: Shannon Wiener Diversity Index (H') of various treatment groups at day 0. None of these values is significantly different from the other ($P>0.05$).

Treatment Group	H'	Mean H'	Std Error
NT1	1.10	1.06	0.05
NT2	0.96		
NT3	1.12		
N1	1.25	1.19	0.06
N2	1.24		
N3	1.07		
CT1	1.24	1.20	0.06
CT2	1.08		
CT3	1.29		
C1	1.16	1.20	0.04
C2	1.15		
C3	1.29		

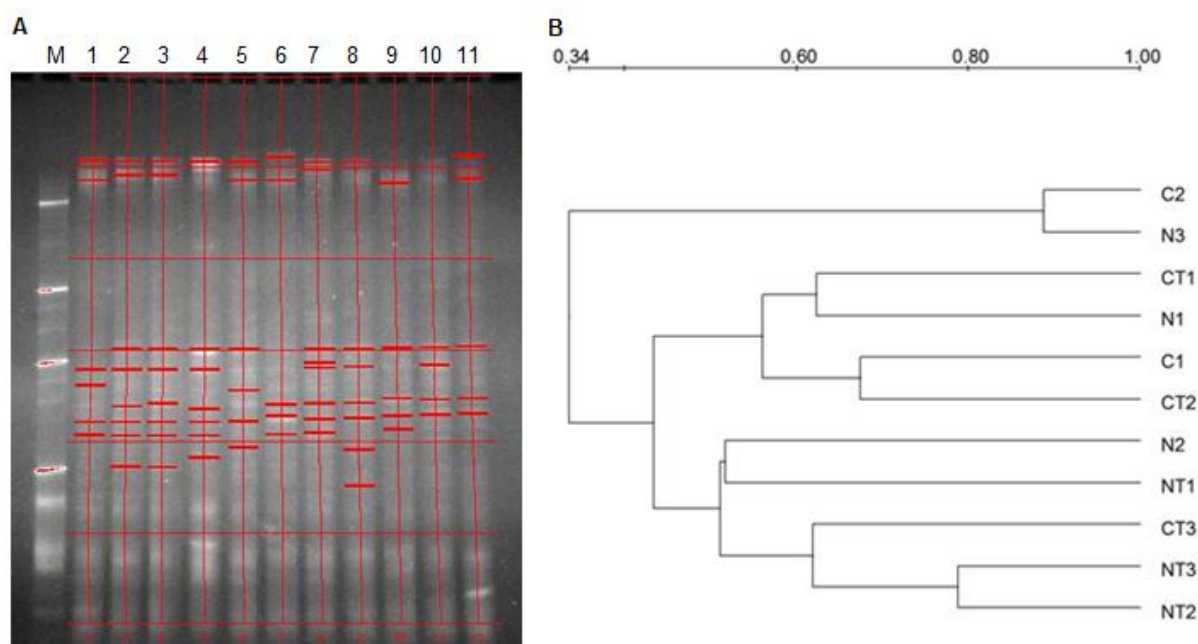


Figure 4.9: DGGE results for all groups at week 4. (A) DGGE with overlay. Red overlay indicates bands recognised and matched using Quantity One software. Lane 1: NT1, lane 2: NT2, lane 3: NT3, lane 4: N1, lane 5: N2, lane 6: N3, lane 7: CT1, lane 8: CT2, lane 9: CT3, lane 10: C1 and lane 11: C2. M represents reference strain marker, identities as shown in Figure 4.4. (B) Dendrogram showing relatedness of bands. Scale bar represents relative distance as Dice coefficients.

Table 4.3: Shannon Wiener Diversity Index (H') of various treatment groups at week 4. None of these values is significantly different from the other ($P>0.05$).

Treatment Group	H'	Mean H'	Std Error
NT1	0.43	0.54	0.06
NT2	0.64		
NT3	0.55		
N1	0.55	0.53	0.04
N2	0.45		
N3	0.59		
CT1	0.52	0.46	0.04
CT2	0.48		
CT3	0.38		
C1	0.32	0.46	
C2	0.61		

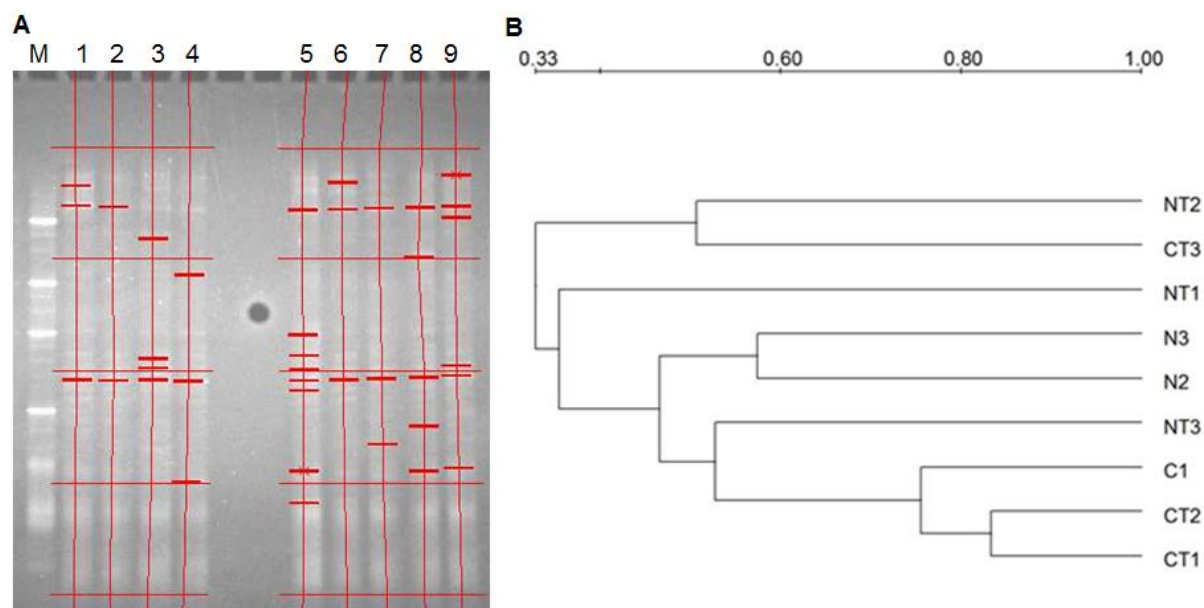


Figure 4.10: DGGE results for all groups at week 9. (A) DGGE with overlay. Red overlay indicates bands recognised and matched using Quantity One software. Lane 1: CT1, lane 2: CT2, lane 3: CT3, lane 4: NT1, lane 5: NT2, lane 6: NT3, lane 7: C1, lane 8: N2 and lane 9: N3. M represents reference strain marker, identities as shown in Figure 4.4. (B) Dendrogram showing relatedness of bands. Scale bar represents relative distance as Dice coefficients.

Table 4.4: Shannon Wiener Diversity Index (H') of various treatment groups at week 9. None of these values is significantly different from the other ($P>0.05$).

Treatment Group	H'	Mean H'	Std Error
NT1	0.44	0.59	0.15
NT2	0.88		
NT3	0.44		
N2	0.66	0.71	0.05
N3	0.75		
CT1	0.46	0.45	0.08
CT2	0.3		
CT3	0.58		
C1	0.43		

six groups treated with probiotics (groups N2, NT1, NT3 and NT2) cluster together in this analysis. At week four there are bands at a similar position to strain 5934.3.1 in groups NT2 and NT3. There are fewer groups at week 4 than at day 0 as a result of the mortality rate at this stage (see chapter three). The number decreases further at week nine for the same reason. The same analysis at week nine shows little clustering of treatment groups in the dendrogram (Figure 4.10), and no statistical similarity of H' values between replicate groups receiving the same treatment (Table 4.4). The matching of bands showed one band at a similar to position to strain P1.2 in group NT2, and one band at a position consistent with strain 136.2.2J in group N3.

4.3.3. DGGE Analysis of Similar Treatments at Different Times

DGGE analysis was performed in order to compare the microbial communities in either tylosin treated or non-tylosin treated birds at different time points. The microbiota at day 0 was compared with that of the same treatment groups at week 4 and week 9.

Figure 4.11A shows a DGGE experiment of samples from tylosin treated groups at day 0 and week 4. The dendrogram constructed from this gel shows that all the day 0 samples cluster together, and all the week 4 samples cluster together, with the exception of CT2 at week 4, which clusters with the day 0 samples. However, no such clustering is seen in the H' values calculated from this DGGE experiment (Table 4.5).

A similar analysis can be seen for non-tylosin treated birds at day 0 and week 4 in Figure 4.12 and Table 4.6. Here, the dendrogram shows clustering of day 0 birds with one another and week 4 birds with one another. Table 4.6 shows that there is no statistically significant difference between the H' values of the birds at these time points.

The DGGE analysis of tylosin treated birds at day 0 and week 9 are shown in Figure 4.13A. The dendrogram of this gel (Figure 4.13B) shows that the day 0 groups cluster together, as do

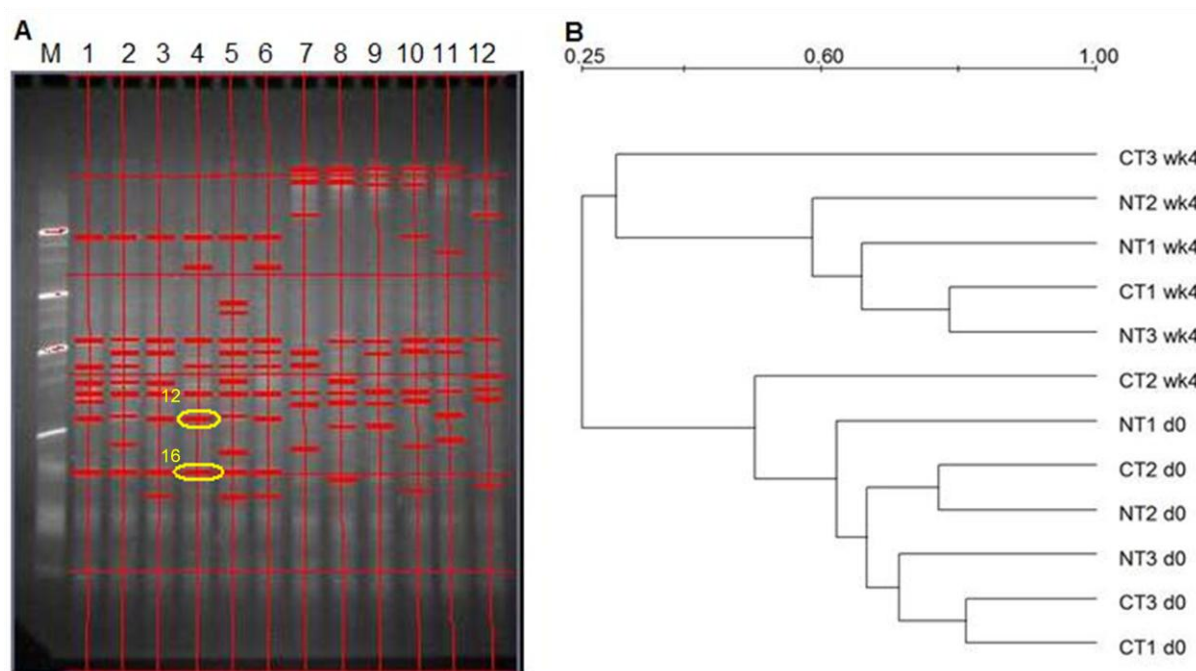


Figure 4.11: DGGE analysis of tylosin treated groups at day 0 and week 4. (A) DGGE with overlay. Red overlay indicates bands recognised and matched using Quantity One software. Day 0 samples are shown in lanes 1-6. Week 4 samples are shown in lanes 7-12. Lane 1: NT1, lane 2: NT2, lane 3: NT3, lane 4: CT1, lane 5: CT2, lane 6: CT3, lane 7: NT1, lane 8: NT2, lane 9: NT3, lane 10: CT1, lane 11: CT2 and lane 12, CT3. M represents reference strain marker, identities as shown in Figure 4.4. Yellow rings indicate bands which were sequenced, the higher called band 12 and the lower called band 16. (B) Dendrogram showing relatedness of bands. Scale bar represents relative distance as Dice coefficients. The annotations d0 and wk4 represent day 0 and week 4 respectively.

Table 4.5: Shannon Wiener Diversity Index (H') of tylosin treated groups at day 0 and week 4. None of these values is significantly different from the other ($P>0.05$).

Treatment Group	H'	Mean H'	Std Error
NT1 d0	0.90	0.95	0.09
NT2 d0	1.00		
NT3 d0	1.11		
CT1 d0	0.77	0.86	0.10
CT2 d0	1.13		
CT3 d0	0.80		
NT1 wk4	0.85		
NT2 wk4	1.08		
NT3 wk4	0.95		
CT1 wk4	0.96		
CT2 wk4	0.71		
CT3 wk4	0.61		

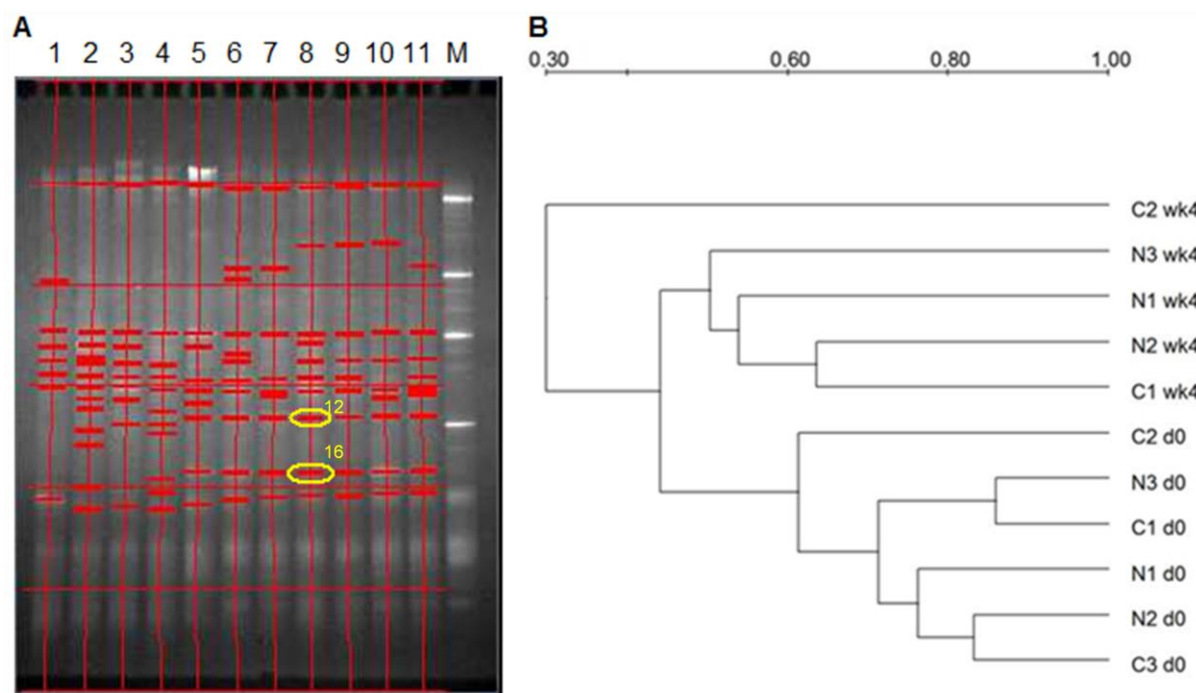


Figure 4.12: DGGE analysis of non-tylosin treated groups at day 0 and week 4. (A) DGGE with overlay. Red overlay indicates bands recognised and matched using Quantity One software. Week 4 samples are shown in lanes 1-6. Day 0 samples are shown in lanes 7-11. Lane 1: C2, lane 2: C1, lane 3: N3, lane 4: N2, lane 5: N1, lane 6: C3, lane 7: C2, lane 8: C1, lane 9: N3, lane 10: N2 and lane 11: N1. M represents reference strain marker, identities as shown in Figure 4.4. Yellow rings indicate bands which were sequenced, the higher called band 12 and the lower called band 16. (B) Dendrogram showing relatedness of bands. Scale bar represents relative distance as Dice coefficients. The annotations d0 and wk4 represent day 0 and week 4 respectively.

Table 4.6: Shannon Wiener Diversity Index (H') of non-tylosin treated groups at day 0 and week 4. None of these values is significantly different from the other ($P>0.05$).

Treatment Group	H'	Mean H'	Std Error
C1 d0	1.38	1.12	0.10
C2 d0	1.12		
C3 d0	1.38		
N1 d0	1.07		
N2 d0	1.25		
N3 d0	1.24		
C1 wk4	1.45	1.12	0.10
C2 wk4	0.86		
N1 wk4	0.95		
N2 wk4	1.14		
N3 wk4	1.18		

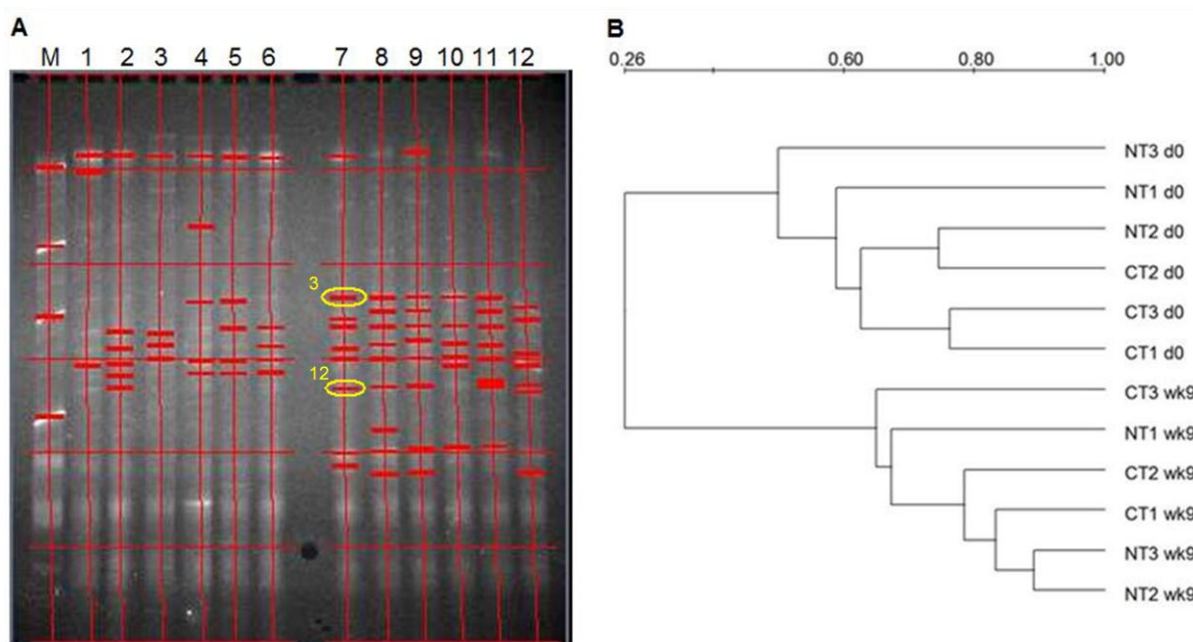


Figure 4.13: DGGE analysis of tylosin treated groups at day 0 and week 9. (A) DGGE with overlay. Red overlay indicates bands recognised and matched using Quantity One software. Week 9 samples are shown in lanes 1-6. Day 0 samples are shown in lanes 7-12. Lane 1: CT1, lane 2: CT2, lane 3: CT3, lane 4: NT1, lane 5: NT2, lane 6: NT3, lane 7: CT1, lane 8: CT2, lane 9: CT3, lane 10: NT1, lane 11: NT2 and lane 12: NT3. M represents reference strain marker, identities as shown in Figure 4.4. Yellow rings indicate bands which were sequenced, the higher called band 3 and the lower called band 12. (B) Dendrogram showing relatedness of bands. Scale bar represents relative distance as Dice coefficients. The annotations d0 and wk9 represent day 0 and week 9 respectively.

Table 4.7: Shannon Wiener Diversity Index (H') of tylosin treated groups at day 0 and week 9. The mean H' values are significantly different ($P=0.037$).

Treatment Group	H'	Mean H'	Std error
CT1 d0	1.10	0.87	0.23
CT2 d0	0.84		
CT3 d0	1.13		
NT1 d0	0.68		
NT2 d0	0.91		
NT3 d0	0.54		
CT1 wk9	0.64	0.60	0.07
CT2 wk9	0.68		
CT3 wk9	0.49		
NT1 wk9	0.62		
NT2 wk9	0.62		
NT3 wk9	0.56		

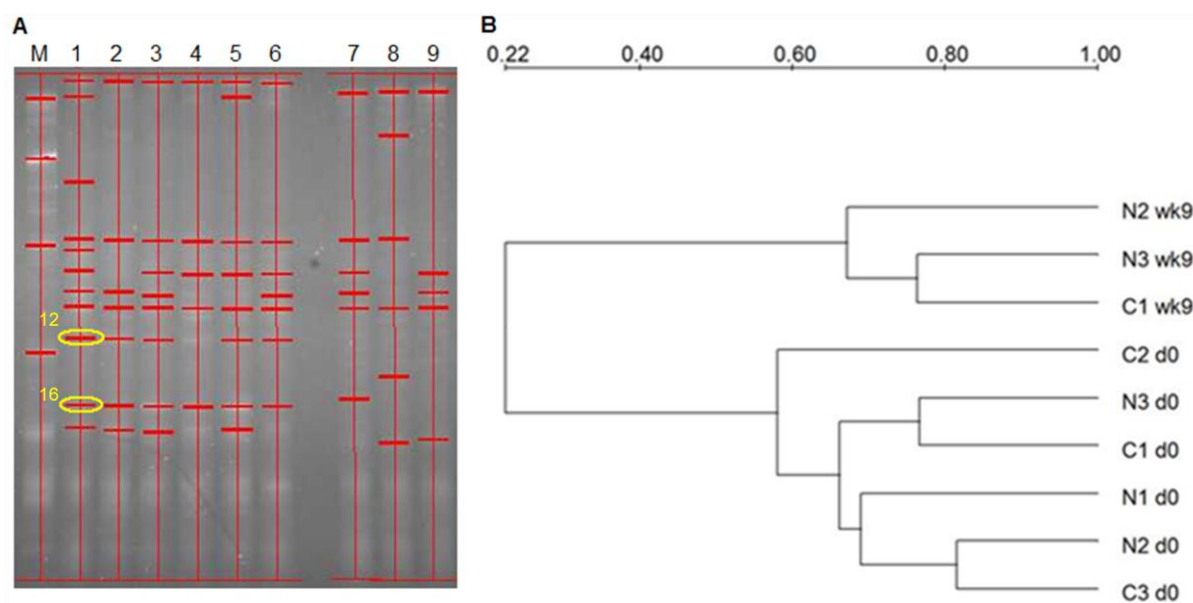


Figure 4.14: DGGE analysis of non-tylosin treated groups at day 0 and week 9. (A) DGGE with overlay. Red overlay indicates bands recognised and matched using Quantity One software. Day 0 samples are shown in lanes 1-6. Week 9 samples are shown in lanes 7-9. Lane 1: C1, lane 2: C2, lane 3: C3, lane 4: N1, lane 5: N2, lane 6: N3, lane 7: C1, lane 8: N2 and lane 9: N3. M represents reference strain marker, identities as shown in Figure 4.4. Yellow rings indicate bands which were sequenced, the higher called band 12 and the lower called band 16. (B) Dendrogram showing relatedness of bands. Scale bar represents relative distance as Dice coefficients. The annotations d0 and wk9 represent day 0 and week 9 respectively.

Table 4.8: Shannon Wiener Diversity Index (H') of non-tylosin treated groups at day 0 and week 9. The mean H' values are significantly different ($P=0.012$).

Treatment Group	H'	Mean H'	Std error
C1 d0	1.53	1.22	0.09
C2 d0	1.11		
C3 d0	1.32		
N1 d0	0.90		
N2 d0	1.33		
N3 d0	1.11		
C1 wk9	0.84	0.87	0.02
N2 wk9	0.91		
N3 wk9	0.87		

the week 9 groups. Table 4.7 shows that the H' values of day 0 groups and week 9 groups are significantly different (P=0.037), the diversity having decreased over time.

Figure 4.14 shows the DGGE analysis of non-tylosin treated birds, comparing the groups at day 0 and at week 9. The dendrogram shows clustering of the day 0 groups with one another, and clustering of week 9 groups with one another. As shown in Table 4.8, the Shannon Wiener Diversity Indices of the day 0 and week 9 birds are significantly different (P=0.012). In these birds, the diversity of the microbiota has decreased over time.

The common bands which were likely to have caused the clustering in the dendrograms or the differences in H' values, were selected by inspection for sequencing, and are indicated by the yellow rings in Figures 4.11, 4.12, 4.13 and 4.14. The H' values, or clustering of a sample in a dendrogram, could be caused by the consistent presence or absence of a band according to sampling time.

Table 4.9: Sequences giving maximum identity to V3 region of 16S rRNA genes excised from DGGE gels. Sequences obtained from uncultured organisms were ignored.

Most Significant Sequence Alignment					
Band No.	Accession No.	Description	Maximum Identity (%)	E Value	Query Coverage (%)
3	NR_026103.1	<i>Clostridium populeti</i> strain 743A	98	2.00x10 ⁻⁸²	99
	AB298768.2	<i>Clostridiaceae bacterium</i> FH052	98	2.00x10 ⁻⁸²	99
	AJ229234.1	<i>Clostridium</i> spp. strain XB90	98	2.00x10 ⁻⁸²	99
	X71853.1	<i>Clostridium populeti</i> ATCC 35295	98	2.00x10 ⁻⁸²	99
	X77839.1	<i>Clostridium polysaccharolyticum</i> DSM 1801	98	2.00x10 ⁻⁸²	99
12	EF025909.1	<i>Clostridium symbiosum</i> strain 69	98	5.00x10 ⁻⁸⁴	100
	EF442669.1	<i>Clostridium symbiosum</i> strain REM001	98	5.00x10 ⁻⁸⁴	100
	NR_025567.1	<i>Clostridium bolteae</i> strain 16351	98	5.00x10 ⁻⁸⁴	100
	M59089.1	<i>Clostridium clostridiiformes</i> ATCC 25537	98	5.00x10 ⁻⁸⁴	100
	M59112.1	<i>Clostridium symbiosum</i> ATCC 14940	98	5.0x10 ⁻⁸⁴	100
16	AJ311620.2	<i>Clostridium hathewayi</i> DSM 13479	97	1.00x10 ⁻⁸⁰	99
	AB525414.1	<i>Eubacterium saburreum</i> strain JCM 11021	97	2.00x10 ⁻⁷⁷	98
	FJ957875.1	<i>Clostridium saccharolyticum</i> strain JPL_23	97	2.00x10 ⁻⁷⁷	98
	FJ957873.1	<i>Clostridium saccharolyticum</i> strain JPL_21	97	2.00x10 ⁻⁷⁷	98
	FM994938.1	<i>Clostridium celerecrescens</i> strain HP2	97	2.00x10 ⁻⁷⁷	98

The bands indicated in the aforementioned figures were named bands 3, 12 and 16, according to the analysis by Quantity One. Band 3, seen in Figure 4.13, is present in the day 0 samples, but not in the week 9 samples. Band 12 is seen in the day 0 samples of tylosin treated and non-tylosin treated birds, and not in samples from week 4 or week 9 birds. Band 16 is present in all birds at day 0, while it is absent in all birds at week 4. Band 16 is absent only in the non-tylosin treated birds at week 9 (and is present in the tylosin treated birds at week 9). Thus, band 16 is only important to the difference between day 0 and week 9 in the non-tylosin treated birds. The sequences from the NCBI database giving maximum identity to the sequences of these bands are shown in Table 4.9. Band 3 was identified as *Clostridium populeti*, while band 12 was identified as *Clostridium symbiosum* and band 16 as *Clostridium hathewayi*.

4.4. Discussion

4.4.1. Optimising DGGE Protocol

Reproducibility of DGGE depends on the PCR amplicons obtained during the preceding PCR steps and the denaturing gradient used during electrophoresis. The amplicons produced by PCR by two polymerase preparations, Kapa Taq and Kapa Readymix, were compared. The products appear similar after PCR using primers Uni-341F and Uni-515R. After the GC clamp is added (by PCR using primers Uni-341F-GC and Uni-515R), there is a visibly smaller amount of amplicon produced by Kapa Readymix in comparison to Kapa Taq. The differing quantities of amplicon in turn affect band intensity of the reference strain marker on a DGGE gel.

The previous comparisons have shown that a large discrepancy in the quality of amplicons, as determined by agarose gel electrophoresis, could affect the intensity of amplicons during DGGE analysis. In order to maximise reproducibility, amplicons were only subjected to DGGE if they had a similar intensity and specificity to the amplicons shown in Figures 4.5 and 4.6. This analysis with a low base pair marker was also used to ensure that the 40 bp GC clamp had been incorporated into the previous amplicons.

The absence of a band for strain 136.3.1 on the DGGE gel using a 45-55% gradient suggests that the melting temperature of the V3 region for this strain is higher than the gradient at the bottom region of the gel (55% denaturant). The use of a wider gradient may allow the analysis of this band. Nevertheless, DGGE on a 45-65% gradient was still unable to visualise a band from this strain. Widening the gradient any more decreased the resolution of area of interest, where the band of the other four strains lie (results not shown). It was therefore decided to limit the analysis to a denaturing gradient of 45-55% for the purposes of this study. In addition to this, previous work has shown that a specific view of the

Bifidobacterium genus can be obtained by using a different primer set during both PCR reactions (Kok *et al.*, 1996; Langendijk *et al.*, 1995). A better analysis of the bifidobacteria within the microbiota may be obtained by performing a separate analysis using such primers. The highly conserved nature of the 16S rRNA among *Bifidobacterium* spp. and the presence of multiple copies of the genes coding for 16S rRNA on the chromosome, could affect analysis of the diversity of these species by DGGE. An alternative gene for amplification and separation by DGGE, the transaldolase gene, has been identified, and may provide more a more accurate reflection of the diversity of the bifidobacteria present in the microbiota (Requena *et al.*, 2002).

This optimisation was undertaken to ensure that all DGGE experiments included in the analysis would be optimal for identifying strains of interest. Assessing the quality of amplicons before DGGE confirm that differences in the intensity of bands observed after DGGE is not due to inefficient PCR.

4.4.2. DGGE Analysis of All Treatment Groups over Time

Five faecal samples from the same treatment group replicates were combined before extraction in order to limit natural variation between individuals, in order to best observe the changes in microbiota caused by the different treatments (Zhou *et al.*, 2007).

DGGE of samples from day zero show bands similar to strains 512.3.1 and P1.2 in non-probiotic fed treatments and all treatments, respectively. As these samples were taken before any treatment began, this represents the baseline microbiota of these ostrich chicks. This data suggests that some of the ostrich chicks may have had *Lactobacillus brevis* and *Enterococcus faecalis* as a part of their microbiota prior to treatment. Analysis at week four shows the presence of bands possibly corresponding to *Lactobacillus oris* in two probiotic and tylosin treated groups. Since this strain was not present in the day zero samples, it is possible that

their presence is due to the *L. oris* strain 5934.3.1 surviving passage through the gastrointestinal tract. DGGE of samples from week nine show the presence of a band consistent with strain P1.2 in group NT2, and a band similar to 136.2.2J in group N3. Strain P1.2 was present in all samples at day zero, but absent at week four. Therefore its presence may be due to probiotic treatment, or due to the presence of this strain in the ostrich chicks' living environment, resulting from the strains' earlier presence in samples at day zero. The presence of bands consistent with strain P1.2 in a probiotic and tylosin treated group may be due to the presence of *E. faecalis* strain P1.2, but the presence of similar strains before treatment began may indicate that this bacterium represents a normal part of the microbiota. The band consistent with strain 136.2.2J was present only at nine weeks. This may indicate that *L. johnsonii* strain 136.2.2J has survived passage through the gastrointestinal tract. While a similar position of bands may indicate the presence of a known species, it should be remembered that DGGE bands migrate according to their melting temperature. Therefore, it is possible for two bands of different sequences, but similar melting temperatures, to co-migrate. The identity of a band would need to be confirmed by excising this band from the gel and sequencing it. Time did not permit identification of the bands as a part of this study. The possibility of co-migrating band species means that the bands should be cloned before sequencing, in order to separate possibly mixed strains. In this case, the presence of more than one species would be detected by sequencing more than one clone from each band (Felske and Osborn, 2005).

The data shows no trend of probiotic strains being present in the probiotic groups while being absent in the control groups. As a whole, there was little detection of bands consistent with the probiotic strains. It is possible that the probiotic strains comprised a relatively small proportion of the microflora, and were therefore undetectable. The first description of the detection capabilities of DGGE was 1% of the population (Muyzer *et al.*, 1993). Later studies

have described the detection level of various DGGE protocols to be 10^4 - 10^8 cfu/ml (Temmerman *et al.*, 2003; Fasoli *et al.*, 2003), depending on the bacterial strain, and the substance that the bacterial DNA was extracted from (Ercolini, 2004). Further studies using DGGE to detect bacteria in ostrich faecal matter should include a study of the detection limit for each strain in ostrich faecal samples. This could be achieved by mixing a known amount of bacteria into a faecal sample before DNA extraction. By processing this sample as normal, and comparing this on a DGGE gel to a sample with no added bacteria, the lower limit of detection can be determined. It has also been shown that using silver staining to visualise DNA instead of ethidium bromide staining can increase the sensitivity of DGGE analysis by a factor of ten (Dewettinck *et al.*, 2001). Alternatively, RNA could be extracted from the faecal samples instead of DNA as starting template. This would show the bacteria within the sample which are most metabolically active (Felske and Osborn, 2005).

In order to ensure that the microbial diversity of all birds was similar before treatment began, the DGGE profiles of different treatment groups were compared at day 0. DGGE analyses of samples taken at day zero show that there is no quantifiable difference between the microbial diversity of the different treatment groups. Specifically, there is no discernable clustering of treatments within the dendrogram. In addition to this, the Shannon Wiener Diversity Indices of the different treatments have no statistical difference to each other. Grouping the H' values into probiotic fed and non-probiotic fed groups also shows no statistically significant difference ($P > 0.05$). According to these two measures of diversity (Dice's coefficient and Shannon Wiener Index), there is no difference in the diversity of the microbiota of ostrich chicks in the different groups prior to administration of the probiotics.

The dendrogram of week four samples did show some clustering of samples by treatment. Groups NT1, CT3, NT3 and NT2 clustered together, along with a non-tylosin treated group, N2. In addition to this, four of the six groups treated with probiotics (N2, NT1, NT3 and

NT2) clustered together at this time point. These two instances of clustering suggest that these treatment groups have somewhat similar bacterial species, but the inclusion of other treatments in this cluster suggests that this similarity is not due to treatment. The other measure of diversity used in this study, the Shannon Wiener Diversity Index, shows that none of the treatment groups has a significantly different diversity ($P>0.05$).

Analysis of diversity by dendrogram at week nine shows that there is no clustering of groups according to treatment. The Shannon Wiener Indices at week nine show that there is no significant difference in diversity between the treatment groups.

The analysis of diversity at three time points during the trial suggests that within each time point, there is little or no difference between any of the treatment groups. The relative similarity of all birds at each time, regardless of treatment, suggests that neither tylosin treatment, probiotic treatment, nor a combination of the two affect the diversity of the microbiota. However, this observation is limited to the amplicons which denature at a point that lies between the 45 and 55% denaturant. A wider range of analysis, employing different denaturing gradients, perhaps complementary methods, may provide additional data which can build on these conclusions.

The visual comparison of DGGE experiments at day zero, week four and week nine shows that the banding patterns are somewhat different. Despite this, previous work has shown that inconsistencies between individual DGGE gels make statistical comparison between gels difficult (Powell *et al.*, 2005). In addition to the limitations associated with sensitivity, DGGE as a method also has limitations with respect to reproducibility, which can arise during genomic DNA extraction, the PCR steps, or due to inconsistencies in denaturing gradient. The formation of heteroduplexes or the complete melting of PCR products, causing a smear of ssDNA could also interfere with the DGGE analysis, if they are not excluded from the analysis correctly (Felske and Osborn, 2005).

To limit the error associated with a comparison between gels, DGGE gels of similar treatment groups at different time points were produced.

4.4.3. DGGE Analysis of Similar Treatments at Different Times

The comparison of the microbiota of ostrich chicks at different time points allows a view of the development of the microbiota, to determine if the microbiota changes significantly as the chicks age. While there is no documented change in the microbiota of ostrich chicks as they age, there is evidence that the microbiota of chickens, the model animal used as defined in chapter one, changes as they age (Lu *et al.*, 2003; Gabriel *et al.*, 2006). The DGGE profiles of chicks at day 0 and week 4 were compared, and profiles at day 0 and week 9 were compared. All profiles which were compared were from chicks which underwent similar treatments, grouped into tylosin treated and non-tylosin treated birds.

Comparison of day 0 with week 4, analysed in a dendrogram, shows that day 0 groups cluster with one another and week 4 groups cluster with one another in the case of both tylosin treated and non-tylosin treated groups. The only exception to this observation was group CT2 at week 4, which clustered with the day 0 tylosin treated groups. In contrast to this analysis using the Dice coefficient and dendrograms, the Shannon Wiener Similarity Indices for these groups were not significantly different at day 0 and week 4. This indicates that these groups have different bacterial species, but there is no change in overall diversity. This is possible because two lanes containing the same number of bands of similar relative intensities will have similar H' values, even if the positions of these bands is different (Gafan *et al.*, 2005).

Analysis of DGGE profiles at day 0 and week 9 show that for both treatments, the dendrogram clusters day 0 birds into one group and week 9 birds into another. The analysis of these profiles using the Shannon Wiener Indices also showed that the groups at day 0 and week 9 were significantly different in their diversities.

These analyses suggest that the microbiota of ostriches changes somewhat between day 0 and week 4, and that it changes more significantly between day 0 and week 9, in the bacterial strains that can be amplified by the primers used and that appear in the denaturing range of 45-55%. This finding is consistent with studies which used DGGE analysis to examine the change in diversity of the microbiota due to age. The composition of the microbiota has been shown to change with age in both the ilea and caeca of young chicks, in the absence of antibiotic or probiotic treatment, using DGGE and similar analysis methods to those used in this study (Hume *et al.*, 2003; Knarreborg *et al.*, 2002; Gong *et al.*, 2008; Lu *et al.*, 2003).

There were two cases where the Shannon Wiener Diversity Indices were significantly different across time points. This was in tylosin treated birds between day 0 and week 9 and in non-tylosin treated birds at the same time points. The birds which were treated with tylosin showed an increase in diversity over time. The ostrich chicks which were not treated with tylosin showed a decrease in diversity over time. The observation of a change in microbial diversity over time during tylosin treatment is consistent with findings in poultry, although this work did not specify whether the change was an increase or a decrease in diversity (Collier *et al.*, 2003). Since the ostrich chicks, which were not treated with tylosin, had a lower percentage survival than those that did receive tylosin treatment (chapter three), this may suggest that a decrease in diversity of the microbiota is associated with a decrease in percentage survival of a treatment group. More analyses would have to be done in order to confirm this observation.

The bands which caused the differences in diversity between time points were of particular interest. Sequence analysis of some of bands 3, 12 and 16 identified them as *Clostridium populeti*, *Clostridium symbiosum* and *Clostridium hathewayi*, respectively. *C. symbiosum* and *C. hathewayi* were both originally isolated from human stool samples (Kaneuchi *et al.*, 1976; Steer *et al.*, 2001), while *C. populeti* is of unknown origin, but has been shown to have

cellulolytic activity (Ren *et al.*, 2007). Band 3 was seen in day 0 ostrich chicks treated with tylosin, but not in week 9 birds. This suggests that *C. populeti* is present in chicks treated with tylosin at day 0, and at week 4, but later, at week 9, is no longer a major member of the microbiota. Band 12 was found in groups of all treatments at day 0, but found in no treatment groups at either week 4 or week 9. This suggests that *C. symbiosum* is a member of the microbiota at day 0, but is lost by week 4 and has not returned by week 9. Since this has occurred in all treatments, the loss may not be due to tylosin sensitivity, and rather due some other reason, such as competition with other strains, which are becoming established. Band 16 was present in all ostrich chicks at day 0 and absent in all birds at week 4. This band was present at week 9 in tylosin treated groups, but absent in non-tylosin treated birds. This suggests that *C. hathewayi* is a part of the microbiota in these birds at day 0, but is lost by week 4 and returns only in the tylosin treated groups at week 9. Since *C. hathewayi* may only re-colonise the GIT when in the presence of tylosin, this may suggest that the strain is resistant to tylosin. It is interesting that the three strains chosen for sequence analysis due to their effect on diversity of the microbiota over time were identified as *Clostridium* spp., and the data suggests that they may all be resistant to tylosin treatment. The presence of Clostridia in the microbiota of ostriches is not unexpected, as work by Matsui *et al.* (2010b) showed that 46 % of sequences obtained from the caecum of an ostrich were classified as falling within the *Clostridium coccoides* group, the *Clostridium leptum* group, or other *Clostridium* groups. In addition to this, studies on chicken microbiota have revealed the presence of *Clostridium* spp. in birds of different ages (Lu *et al.*, 2003). The analyses presented here show that no significant effect of tylosin treatment on the microbiota could be seen at any one time point, but the effect of time on the diversity of the microbiota in birds receiving all treatments was noted. The change in diversity could be attributed to the transient presence of three *Clostridium* spp., which seem to be resistant to tylosin treatment. The difference in microbial

diversity over time should be further explored by sequencing more DGGE bands important to this difference in diversity, and comparing the microbiota at more time points by DGGE analysis. While the presence of probiotic strains in the microbiota could not be shown conclusively using the methods shown here, this does not mean that these strains are not present in the microbiota at low levels. In order to determine if this is the case, the detection limit of each probiotic strain in ostrich faecal material should be established.

CHAPTER 5

GENERAL CONCLUSIONS

A limiting factor in the farming of ostrich chicks is chick mortality, which is associated with pathogenic infections within the gastrointestinal tract. A lack of a well established microbiota can render a chick more susceptible to infection, and thus the problem could be addressed by establishing the correct microbiota via the use of probiotics. An ostrich specific probiotic has yet to be developed.

To this end, five strains isolated from healthy adult ostriches, partially characterised in previous work (Juste-Poinapen, 2007), were conclusively identified and characterised further. These strains were then included in an *in vivo* feeding trial, to determine their efficacy in terms of chick mortality, weight gain, and modulation of the composition of the microbiota.

Strain P1.2 was identified as *Enterococcus faecalis* using biochemical tests and sequencing of the 16S rRNA gene and 16S – 23S intergenic spacer region. The identity of strain 5934.3.1 was confirmed to be *Lactobacillus oris* by sequencing of the 16S – 23S intergenic spacer region.

Strains 5934.3.1, P1.2, 136.3.1 and 512.3.1 were assayed for their adhesion to a porcine mucin agar. The adhesion of these strains was compared to that of *Lactobacillus rhamnosus* LGG, and it was concluded that strains 512.3.1 and 5934.3.1 had the best adhesion capabilities of the four selected strains which were assayed. These two strains were then screened for genes which may confer adhesion abilities.

The *mapA* gene was screened for using degenerate and specific PCR primers, while the *msa* gene was screened for using specific primers designed to the *msa* gene from *L. plantarum* WCFS1 and *L. brevis* ATCC 367. These genes were not detected using these methods. No

msa gene could be detected in strain 512.3.1 using Southern hybridisation with a probe generated from *L. brevis* ATCC 367.

The five selected strains belong to species which have previously been used as probiotics, and two of these strains adhere well to porcine mucin agar, suggesting that they will adhere to ostrich intestinal mucins. The additional probiotic characteristics of these strains (Juste-Poinapen, 2007) in combination with results from this work indicate that the five selected strains have characteristics desirable in probiotic strains (Patterson and Burkholder, 2003). Therefore these strains were included in a probiotic feeding trial in ostrich chicks.

Random amplification of polymorphic DNA (RAPD) was investigated as a method for detecting these probiotics in ostrich faecal samples from a pilot feeding trial. None of the strains were detected in faecal samples after probiotic feeding began. In order to obtain meaningful information about the frequency of occurrence of strains, more colonies should be selected for RAPD analysis from each sample. Takahashi *et al.* (2007) used 91-126 isolates from each sample in order to determine the frequency of presence of isolates, and the use of several hundred isolates is suggested for reliable statistical analysis of such a study (Vaughan *et al.*, 2000). While each strain did produce a distinct RAPD profile, this method may prove cumbersome for future trials, due to the amount of culture required, and it was decided to analyse the trials performed in this study using other methods.

A feeding trial was conducted using four different treatments of three-day-old ostrich chicks. The chicks receiving probiotic treatment of a mixture of the five selected strains gained more weight than the chicks on other treatments. Unfortunately, only two replicate groups receiving this treatment remained when this trend appeared, preventing any statistical analysis of this observation.

A large drop in the survival of chicks was observed during the first four weeks of life. This is in accordance with the findings of Cloete *et al.* (2001), who observed the most fatalities of ostrich chicks during the first four weeks. The survival of chicks was improved by treatment with tylosin.

Culture of the faecal samples taken from this trial showed a change in the culturable gut microbiota after four weeks. Specifically, titres of *Clostridium* spp. dropped and then remained low after four weeks. This coincided with the observed drop in survival of chicks not receiving tylosin, but no conclusions could be drawn about the nature of the relationship of these two occurrences. The drop in titres of *Clostridium* spp. in surviving birds may have contributed to their survival.

The feeding trial was analysed by DGGE. While no treatment-specific effects were observed at any one time point during the trial, some age-dependent effects on the microbiota were noted. Dendrograms constructed from gel images showed that birds of all treatments at day 0 and week 4 clustered according to age. This indicates a difference in number and placement of bands in a DGGE profile. The same clustering was seen in dendrograms of birds at day 0 and week 9. In addition to this, birds at day 0 and week 9 had significantly different Shannon Wiener Diversity Indices, indicating difference in number and intensity of bands in the DGGE profiles. This suggests that the unculturable microbiota develops as the ostrich chicks age, a finding that is consistent with the microbiota of poultry (Lu *et al.*, 2003; Gabriel *et al.*, 2006).

Three bands, which contributed to the change in clustering of dendrograms, or in diversity, as indicated by Shannon Wiener Indices, were sequenced. This showed that *Clostridium populeti* is present in the microbiota until week 9, *Clostridium symbiosum* is present at day 0, but lost after this point and *Clostridium hathewayi* is a member of the microbiota at day 0, is lost at week 4 and returns in tylosin treated birds at week 9. *Clostridium* spp. have also been

detected in the microbiota of chickens (Lu *et al.*, 2003), and sequencing of a 16S rRNA gene library constructed from ostrich caeca has shown some *Clostridium* spp. to be the predominant detected bacteria (Matsui *et al.*, 2010b).

Future Work

The proteins responsible for adhesion of strains 512.3.1 and 5934.3.1 to intestinal mucin have not yet been identified. Candidates which have been implicated in adhesion of other *Lactobacillus* spp. to mucins, but have not been included in this work are Mub, GroEL and EF-Tu (Vélez *et al.*, 2007). Screening for the genes encoding Mub proteins, and for the Gro-EL and EF-Tu proteins on the cell surface may reveal the proteins involved in the adhesion of these strains to mucin.

Some modification of the protocol of future probiotic feeding may enable observation of changes in weight gain during the period of maximum growth and identifying the causative agent of any observed enteritis. Extending the duration of future feeding trials will allow observation of the period during which ostriches experience their maximum weight gain, which is between 92 and 175 days of age (Cooper and Mahrose, 2004). Culture of the intestinal contents of the deceased birds may indicate any pathogens which may have caused any fatal enteritis. The immune status of the gut epithelium could also be monitored to further elucidate the effects of the probiotics. This will be difficult, as the mechanisms of immune function have not yet been fully characterised (Spinu *et al.*, 1999), even though the immune status of ostriches has been measured using assays of cell mediated and humoral immune responses (Bonato *et al.*, 2009).

DGGE analysis of faecal samples may be aided by determining the limit of detection of the selected strains by this method. Analysis of faecal samples by other culture-independent

methods such as fluorescent *in situ* hybridisation and sequencing of a genomic library constructed from the 16S rRNA genes of microbiota.

This work has confirmed that, as in the microbiota of poultry, the microbiota of ostrich chicks changes with age, regardless of probiotic treatment. The persistence and effect of the five selected strains on the microbiota may be detected in future feeding trials using the recommendations outlined here. Overall, the findings presented here contribute to the body of knowledge on the microbiota of ostrich chicks, a field where much research is yet to be done.

LITERATURE CITED

Abbas Hilmi, H. T., Surakka, A., Apajalahti, J., and Saris, P. E. (2007). Identification of the most abundant *Lactobacillus* species in the crop of 1- and 5-week-old broiler chickens. *Applied and Environmental Microbiology* **73**: 7867-7873.

Aganga, A. A., Aganga, A. O., and Omphile, U. J. (2003). Ostrich feeding and nutrition. *Pakistan Journal of Nutrition* **2**: 60-67.

Altschul, S., Madden, T., Schäffer, A., Zhang, J., Miller, W., and Lipman, D. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Antikainen, J., Korhonen, T. K., Kuparinen, V., Toba, T., and Roos, S. (2009). Surface proteins of *Lactobacillus* involved in host interactions. In *Lactobacillus* molecular biology: From genomics to probiotics. Å. Ljungh and T. Wadström (Norfolk, UK: Caister Academic Press), pp. 95-114.

Apajalahti, J., and Kettunen, A. (2006). Microbes of the chicken gastrointestinal tract. In *Avian gut function in health and disease*; G. C. Perry (Poultry Science Symposium Series), pp. 124-137.

Armitage, P., Walden, R., and Draper, J. (1988). Vectors for the transformation of plant cells using *Agrobacterium*. In *Plant genetic transformation and gene expression, A laboratory manual* (Blackwell Scientific Publications Ltd, Oxford).

Axelsson, M. A., Asker, N., and Hansson, G. C. (1998). O-Glycosylated MUC2 monomer and dimer from LS 174T cells are water-soluble, whereas larger MUC2 species formed early during biosynthesis are insoluble and contain nonreducible intermolecular bonds. *The Journal of Biological Chemistry* **273**: 18864 -18870.

Becquet, P. (2003). EU assessment of Enterococci as feed additives. *International Journal of Food Microbiology* **88**: 247-254.

Bergonzelli, G. E., Granato, D., Pridmore, R. D., Marvin-Guy, L. F., Donnicola, D., and Corthésy-Theulaz, I. E. (2006). GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: Potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. *Infection and Immunity* **74**: 425-434.

Boekhorst, J., Helmer, Q., Kleerebezem, M., and Siezen, R. J. (2006). Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria. *Microbiology* **152**: 273-280.

Bonato, M., Evans, M. R., Hasselquist, D., Cloete, S. W., and Cherry, M. I. (2009). Growth rate and hatching date in ostrich chicks reflect humoral but not cell-mediated immune function. *Behavioral Ecology and Sociobiology* **64**: 183-191.

Brisbin, J. T., Gong, J., and Sharif, S. (2008). Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Animal Health Research Reviews* **9**: 101-110.

Canchaya, C., Claesson, M. J., Fitzgerald, G. F., van Sinderen, D., and O'Toole, P. W. (2006). Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. *Microbiology* **152**: 3185-3196.

Carbajo, E. (2006). Ostrich production to mature in more countries. *World Poultry* **22**: 24-26.

Carlstedt, I., Herrmann, A., Karlsson, H., Sheehan, J., Fransson, L., and Hansson, G. C. (1993). Characterization of two different glycosylated domains from the insoluble mucin complex of rat small intestine. *The Journal of Biological Chemistry* **268**: 18771-18781.

Cloete, S. W., Lambrechts, H., Punt, K., and Brand, Z. (2001). Factors related to high levels of ostrich chick mortality from hatching to 90 days of age in an intensive rearing system. *Journal of the South African Veterinary Association* **72**: 197-202.

Coconnier, M. H., Klaenhammer, T. R., Kernéis, S., Bernet, M. F., and Servin, A. L. (1992). Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. *Applied and Environmental Microbiology* **58**: 2034-2039.

Collado, M. C., Surono, I., Meriluoto, J., and Salminen, S. (2007). Indigenous dadih lactic acid bacteria: Cell-surface properties and interactions with pathogens. *Journal of Food Science* **72**: M89-93.

Collier, C. T., Van Der Klis, J. D., Deplancke, B., Anderson, D. B., and Gaskins, H. R. (2003). Effects of tylosin on bacterial mucolysis, *Clostridium perfringens* colonization, and intestinal barrier function in a chick model of necrotic enteritis. *Antimicrobial Agents and Chemotherapy* **47**: 3311-3317.

Cooper, R. G., Kennou Sebei, S., Horbańczuk, J. O., Villegas-Vizcaíno, R., Mohammed, A. E., and Mahrose, K. M. (2010). Wild ostrich (*Struthio camelus*) ecology and physiology. *Tropical Animal Health and Production* **42**: 363-373.

Cooper, R. G., and Mahrose, K. M. (2004). Anatomy and physiology of the gastrointestinal tract and growth curves of the ostrich (*Struthio camelus*). *Animal Science Journal* **75**: 491-498.

De Angelis, M., Siragusa, S., Caputo, L., Ragni, A., Burzigotti, R., and Gobbetti, M. (2007). Survival and persistence of *Lactobacillus plantarum* 4.1 and *Lactobacillus reuteri* 3S7 in the gastrointestinal tract of pigs. *Veterinary Microbiology* **123**: 133-144.

Dewettinck, T., Hulsbosch, W., Van Hege, K., Top, E., and Verstraete, W. (2001). Molecular fingerprinting of bacterial populations in groundwater and bottled mineral water. *Applied Microbiology and Biotechnology* **57**: 412-418.

Domann, E., Hain, T., Ghai, R., Billion, A., Kuenne, C., Zimmermann, K., and Chakraborty, T. (2007). Comparative genomic analysis for the presence of potential enterococcal virulence factors in the probiotic *Enterococcus faecalis* strain Symbioflor 1. *International Journal of Medical Microbiology* **297**: 533-539.

Donoghue, A. M., Farnell, M. B., Cole, K., and Donghue, D. J. (2006). Mechanisms of pathogen control in the avian gastrointestinal tract. In *Avian gut function in health and disease*; G. C. Perry (Poultry Science Symposium Series), pp. 138-155.

Edelman, S., Westerlund-Wikström, B., Leskelä, S., Kettunen, H., Rautonen, N., Apajalahti, J., and Korhonen, T. K. (2002). *In vitro* adhesion specificity of indigenous Lactobacilli within the avian intestinal tract. *Applied and Environmental Microbiology* **68**: 5155-5159.

Ehrmann, M. A., Kurzak, P., Bauer, J., and Vogel, R. F. (2002). Characterization of Lactobacilli towards their use as probiotic adjuncts in poultry. *Journal of Applied Microbiology* **92**: 966-975.

Eichner, C. A., Erb, R. W., Timmis, K. N., and Wagner-Döbler, I. (1999). Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community. *Applied and Environmental Microbiology* **65**: 102-109.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods* **56**: 297-314.

Fasoli, S., Marzotto, M., Rizzotti, L., Rossi, F., Dellaglio, F., and Torriani, S. (2003). Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *International Journal of Food Microbiology* **82**: 59-70.

Felske, A., and Osborn, M. (2005). DNA fingerprinting of microbial communities. In *Molecular Microbial Ecology* M. Osborn and C. Smith (New York: Taylor and Francis), pp. 65-90.

Fujiwara, S., Seto, Y., Kimura, a., and Hashiba, H. (2001). Establishment of orally-administered *Lactobacillus gasseri* SBT2055SR in the gastrointestinal tract of humans and its influence on intestinal microflora and metabolism. *Journal of Applied Microbiology* **90**: 343-352.

Gabriel, I., Lessire, M., Mallet, S., and Guillot, J. (2006). Microflora of the digestive tract: Critical factors and consequences for poultry. *World's Poultry Science Journal* **62**: 499-511.

Gafan, G. P., Lucas, V. S., Roberts, G. J., Petrie, A., Wilson, M., and Spratt, D. A. (2005). Statistical analyses of complex denaturing gradient gel electrophoresis profiles. *Journal of Clinical Microbiology* **43**: 3971-3978.

Gomes, A. M., and Malcata, F. X. (1999). *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends in Food Science & Technology* **10**: 139-157.

Gong, J., Forster, R. J., Yu, H., Chambers, J. R., Sabour, P. M., Wheatcroft, R., and Chen, S. (2002). Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. *FEMS Microbiology Letters* **208**: 1-7.

Gong, J., Si, W., Forster, R. J., Huang, R., Yu, H., Yin, Y., Yang, C., and Han, Y. (2007). 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: From crops to ceca. *FEMS Microbiology Ecology* **59**: 147-157.

Gong, J., Yu, H., Liu, T., Gill, J. J., Chambers, J. R., Wheatcroft, R., and Sabour, P. M. (2008). Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. *Journal of Applied Microbiology* **104**: 1372-1382.

Granato, D., Bergonzelli, G. E., Pridmore, R. D., Marvin, L., Rouvet, M., and Corthésy-Theulaz, I. E. (2004). Cell surface-associated Elongation Factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infection and Immunity* **72**: 2160-2169.

Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., and Brassart, D. (1999). Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Applied and Environmental Microbiology* **65**: 1071-1077.

Greenhill, N. (2007). Development of potential probiotics for use in ostrich chicks (*Struthio camelus*). B.Sc. (Hons) thesis, University of Cape Town.

Gross, G., Snel, J., Boekhorst, J., Smits, M. A., and Kleerebezem, M. (2010). Biodiversity of mannose-specific adhesion in *Lactobacillus plantarum* revisited: Strain-specific domain composition of the mannose-adhesin. *Beneficial Microbes* **1**: 61-66.

Gross, G., van Der Meulen, J., Snel, J., Meer, R. V., Kleerebezem, M., Niewold, T. A., Hulst, M. M., and Smits, M. A. (2008). Mannose-specific interaction of *Lactobacillus plantarum* with porcine jejunal epithelium. *FEMS Immunology and Medical Microbiology* **54**: 215-223.

Gérard, P., Brézillon, C., Quéré, F., Salmon, A., and Rabot, S. (2008). Characterization of cecal microbiota and response to an orally administered *Lactobacillus* probiotic strain in the broiler chicken. *Journal of Molecular Microbiology and Biotechnology* **14**: 115-122.

Haghighi, H. R., Gong, J., Gyles, C. L., Hayes, M. A., Zhou, H., Sanei, B., Chambers, J. R., and Sharif, S. (2006). Probiotics stimulate production of natural antibodies in chickens. *Clinical and Vaccine Immunology* **13**: 975-980.

Hamscher, G., Limsuwan, S., Tansakul, N., and Kietzmann, M. (2006). Quantitative analysis of tylosin in eggs by high performance liquid chromatography with electrospray ionization tandem mass spectrometry: Residue depletion kinetics after administration via feed and drinking water in laying hens. *Journal of Agricultural and Food Chemistry* **54**: 9017-9023.

Hauschild, A., Hilsheimer, R., and Griffith, D. (1974). Enumeration of fecal *Clostridium perfringens* spores in egg yolk-free tryptose-sulphite-cycloserine agar. *Applied Microbiology* **27**: 527-530.

Havenith, C. E., Seegers, J. F., and Pouwels, P. H. (2002). Gut-associated Lactobacilli for oral immunisation. *Food Research International* **35**: 151-163.

Huchzermeyer, F. W. (2002). Diseases of farmed crocodiles and ostriches. *Revue scientifique et technique (International Office of Epizootics)* **21**: 265-276.

Hume, M. E., Kubena, L. F., Edrington, T. S., Donskey, C. J., Ricke, S. C., and Nisbet, D. J. (2003). Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. *Poultry Science* **82**: 1100-1107.

Ish-Horowicz, D., and Burke, J. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Research* **9**: 2989-2998.

Jacobsen, C. N., Rosenfeldt Nielsen, V., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Pæregaard, A., Sandström, B., Tvede, M., and Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonisation ability of five selected strains in humans. *Applied and Environmental Microbiology* **65**: 4949-4956.

Jennes, W., Dicks, L. M., and Verwoerd, D. J. (2000). Enterocin 012, a bacteriocin produced by *Enterococcus gallinarum* isolated from the intestinal tract of ostrich. *Journal of Applied Microbiology* **88**: 349-357.

Jonsson, H., Ström, E., and Roos, S. (2001). Addition of mucin to the growth medium triggers mucus-binding activity in different strains of *Lactobacillus reuteri* in vitro. *FEMS Microbiology Letters* **204**: 19-22.

Juste-Poinapen, M. S. N. (2007). Evaluation and characterisation of probiotics for use in the ostrich industry. M.Sc. thesis, University of Cape Town.

Kaneuchi, C., Watanabe, K., Terada, A., Benno, Y., and Mitsuoka, T. (1976). Taxonomic study of *Bacteroides clostridiiformis* subsp. *clostridiiformis* (Burri and Ankersmit) Holdeman and Moore and of related organisms: Proposal of *Clostridium clostridiiformis* (Burri and Ankersmit) comb. nov. and *Clostridium symbiosum* (Stevens) comb. nov. *International Journal of Systematic Bacteriology* **26**: 195-204.

Kapa Biosystems (2010). Kapa2G robust PCR kits brochure. [Online] Available at: www.kapabiosystems.co.za/products/name/kapa2g-robust-pcr-kits/ [21 March 2010].

Kelly, D., and Conway, S. (2005). Bacterial modulation of mucosal innate immunity. *Molecular Immunology* **42**: 895-901.

Kennou Sebei, S., and Bergaoui, R. (2009). Ostriches' reproduction behaviour and mastery of natural incubation under farming conditions. *Tropical Animal Health and Production* **41**: 353-361.

Kirjavainen, P. V., Ouwehand, A. C., Isolauri, E., and Salminen, S. J. (1998). The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiology Letters* **167**: 185-189.

Knarreborg, A., Simon, M. A., Engberg, R. M., Jensen, B. B., and Tannock, G. W. (2002). Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Applied and Environmental Microbiology* **68**: 5918-5924.

Kok, R. G., De Waal, A., Schut, F., Welling, G. W., Weenk, G., and Hellingwerf, K. J. (1996). Specific detection and analysis of a probiotic *Bifidobacterium* strain in infant feces. *Applied and Environmental Microbiology* **62**: 3668-3672.

Kullin, B. (2010). Personal communication.

La Ragione, R. M., Narbad, A., Gasson, M. J., and Woodward, M. J. (2004). *In vivo* characterization of *Lactobacillus johnsonii* FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. *Letters in Applied Microbiology* **38**: 197-205.

Lane, D. (1991). 16S/23S rRNA sequencing. In *Nucleic acids techniques in bacterial systematics*, E. Stackebrandt and M. Goodfellow (New York: Wiley), pp. 115-175.

Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. E., Wilkinson, M. H., and Welling, G. W. (1995). Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Applied and Environmental Microbiology* **61**: 3069-3075.

Lidell, M. E., Johansson, M. E., and Hansson, G. C. (2003). An autocatalytic cleavage in the C terminus of the human MUC2 mucin occurs at the low pH of the late secretory pathway. *Journal of Biological Chemistry* **278**: 13944 -13951.

Lidell, M. E., Moncada, D. M., Chadee, K., and Hansson, G. C. (2006). *Entamoeba histolytica* cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 9298-9303.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J., and Lee, M. D. (2003). Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology* **69**: 6816-6824.

Macías-Rodríguez, M. E., Zagorec, M., Ascencio, F., Vázquez-Juárez, R., and Rojas, M. (2009). *Lactobacillus fermentum* BCS87 expresses mucus- and mucin-binding proteins on the cell surface. *Journal of Applied Microbiology* **107**: 1866-1874.

Mahenthiralingam, E., Marchbank, A., Drevinek, P., Garaiova, I., and Plummer, S. (2009). Use of colony-based bacterial strain typing for tracking the fate of *Lactobacillus* strains during human consumption. *BMC Microbiology* **9**: 251-266.

Matsui, H., Ban-Tokuda, T., and Wakita, M. (2010a). Detection of fiber-digesting bacteria in the ceca of ostrich using specific primer sets. *Current Microbiology*. **60**: 112-116.

Matsui, H., Kato, Y., Chikaraishi, T., Moritani, M., Ban-Tokuda, T., and Wakita, M. (2010b). Microbial diversity in ostrich ceca as revealed by 16S ribosomal RNA gene clone library and detection of novel *Fibrobacter* species. *Anaerobe* **16**: 83-93.

Matsumoto, M., Tani, H., Ono, H., Ohishi, H., and Benno, Y. (2002). Adhesive property of *Bifidobacterium lactis* LKM512 and predominant bacteria of intestinal microflora to human intestinal mucin. *Current Microbiology* **44**: 212-215.

Mead, G. C. (2000). Prospects for 'competitive exclusion' treatment to control *Salmonellas* and other foodborne pathogens in poultry. *The Veterinary Journal* **159**: 111-123.

Miyoshi, Y., Okada, S., Uchimura, T., and Satoh, E. (2006). A mucus adhesion promoting protein, MapA, mediates the adhesion of *Lactobacillus reuteri* to Caco-2 human intestinal epithelial cells. *Bioscience, Biotechnology and Biochemistry* **70**: 1622-1628.

Mountzouris, K. C., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G., and Fegeros, K. (2007). Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poultry Science* **86**: 309-317.

Muyzer, G., De Waal, E. C., and Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695-700.

Muñoz-Provencio, D., Llopis, M., Antolín, M., de Torres, I., Guarner, F., Pérez-Martínez, G., and Monedero, V. (2009). Adhesion properties of *Lactobacillus casei* strains to resected intestinal fragments and components of the extracellular matrix. *Archives of Microbiology* **191**: 153-161.

Nakamura, A., Ota, Y., Mizukami, A., Ito, T., Ngwai, Y. B., and Adachi, Y. (2002). Evaluation of Aviguard, a commercial competitive exclusion product for efficacy and after-effect on the antibody response of chicks to *Salmonella*. *Poultry Science* **81**: 1653-1660.

Netherwood, T., Gilbert, H. J., Parker, D. S., and O'Donnell, A. G. (1999). Probiotics shown to change bacterial community structure in the avian gastrointestinal tract. *Applied and Environmental Microbiology* **65**: 5134-5138.

Norin, E., Jernberg, C., Nilsson, H., and Engstrad, L. (2009). Studies of the microflora by traditional, functional and molecular techniques. In *Lactobacillus* molecular biology: From genomics to probiotics. Å. Ljungh and T. Wadström (Norfolk, UK: Caister Academic Press), pp. 83-94.

O'Sullivan, G. C., Kelly, P., O'Halloran, S., Collins, C., Collins, J. K., Dunne, C., and Shanahan, F. (2005). Probiotics: An emerging therapy. *Current Pharmaceutical Design* **11**: 3-10.

Ouwehand, A. C., Isolauri, E., Kirjavainen, P. V., Tölkkö, S., and Salminen, S. J. (2000). The mucus binding of *Bifidobacterium lactis* Bb12 is enhanced in the presence of

Lactobacillus GG and *Lact. delbrueckii* subsp. *bulgaricus*. Letters in Applied Microbiology **30**: 10-13.

Ouwehand, A. C., Niemi, P., and Salminen, S. J. (1999). The normal faecal microflora does not affect the adhesion of probiotic bacteria in vitro. FEMS Microbiology Letters **177**: 35-38.

Pascual, M., Hugas, M., Badiola, J. I., Monfort, J. M., and Garriga, M. (1999). *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonisation in chickens. Applied and Environmental Microbiology **65**: 4981-4986.

Paterson, G. K., and Mitchell, T. J. (2004). The biology of Gram-positive sortase enzymes. Trends in Microbiology **12**: 89-95.

Patterson, J. A., and Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. Poultry Science **82**: 627-631.

Powell, S. M., Riddle, M. J., Snape, I., and Stark, J. S. (2005). Location and DGGE methodology can influence interpretation of field experimental studies on the response to hydrocarbons by Antarctic benthic microbial community. Antarctic Science **17**: 353-360.

Prescott, L. M., Harley, J. P., and Klein, D. A. (2005). Bacteria: The Proteobacteria. In Microbiology (McGraw-Hill), pp. 494-495.

Pretzer, G., Snel, J., Molenaar, D., Wiersma, A., Bron, P. A., Lambert, J., de Vos, W. M., van der Meer, R., Smits, M. A., and Kleerebezem, M. (2005). Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. Journal of Bacteriology **187**: 6128-6136.

Ramiah, K., van Reenen, C. A., and Dicks, L. M. (2007). Expression of the mucus adhesion genes *Mub* and *MapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* of *Lactobacillus plantarum* 423, monitored with real-time PCR. International Journal of Food Microbiology **116**: 405 - 409.

Reid, G. (1999). The scientific basis for probiotic strains of *Lactobacillus*. Applied and Environmental Microbiology **65**: 3763-3766.

Reid, S. J. (2010). Personal communication.

Ren, Z., Ward, T. E., Logan, B. E., and Regan, J. M. (2007). Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic *Clostridium* species. Journal of Applied Microbiology **103**: 2258-2266.

Requena, T., Burton, J., Matsuki, T., Munro, K., Simon, M. A., Tanaka, R., Watanabe, K., and Tannock, G. W. (2002). Identification, detection, and enumeration of human *Bifidobacterium* species by targeting the transaldolase gene. Applied and Environmental Microbiology **68**: 2420-2427.

Rinkinen, M., Westermarck, E., Salminen, S., and Ouwehand, A. C. (2003). Absence of host specificity for in vitro adhesion of probiotic lactic acid bacteria to intestinal mucus. *Veterinary Microbiology* **97**: 55-61.

Rochet, V., Rigottier-Gois, L., Sutren, M., Krementschi M. N., Andrieux, C., Furet, J. P., Tailliez P., Levenez, F., Mogenet A., Bresson, J. L., Méance, S., Cayuela, C., Leplingard A., and Doré, J. (2006). Effects of orally administered *Lactobacillus casei* DN-114 001 on the composition or activities of the dominant faecal microbiota in healthy humans. *British Journal of Nutrition* **95**: 421-429.

Rojas, M., Ascencio, F., and Conway, P. L. (2002). Purification and characterization of a surface protein from *Lactobacillus fermentum* 104R that binds to small intestinal mucus and gastric mucin. *Applied and Environmental Microbiology* **68**: 2330-2336.

Roos, S., Karner, F., Axelsson, L., and Jonsson, H. (2000). *Lactobacillus mucosae* sp. nov., a new species with *in vitro* mucus-binding activity isolated from pig intestine. *International Journal of Systematic and Evolutionary Microbiology* **50**: 251-258.

Roos, S., and Jonsson, H. (2002). A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology* **148**: 433-442.

Salminen, S. von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W. M., Fondén, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S. E., and Mattila-Sandholm, T. (1998). Demonstration of safety of probiotics - a review. *International Journal of Food Microbiology* **44**: 93-106.

Sambrook, J., Fritsch, E., and Maniatis, T. (2001). Molecular cloning: A cloning laboratory manual 3rd Edition. (Cold Spring Harbor, USA: Cold Spring Harbor Laboratory Press).

Samli, H. E., Senkoylu, N., Koc, F., Kanter, M., and Agma, A. (2007). Effects of *Enterococcus faecium* and dried whey on broiler performance, gut histomorphology and intestinal microbiota. *Archives of Animal Nutrition* **61**: 42-49.

Shannon, C., and Weaver, W. (1971). The mathematical theory of communication (Urbana, Illinois: University of Illinois Press).

Shivaprasad, H. L. (1993). Neonatal mortality in ostriches: An overview of possible causes. In *Proceedings of the Association of Avian Veterinarians*, pp. 282-293.

Spinu, M., Spinu, O., and Degen, A. (1999). Haematological and immunological variables in a domesticated and wild subspecies of ostrich (*Struthio camelus*). *British Poultry Science* **40**: 613-618.

Steer, T., Collins, M. D., Gibson, G. R., Hippe, H., and Lawson, P. A. (2001). *Clostridium hathewayi* sp. nov., from human faeces. *Systematic and Applied Microbiology* **24**: 353-357.

Takahashi, S., Egawa, Y., Simojo, N., Tsukahara, T., and Ushida, K. (2007). Oral administration of *Lactobacillus plantarum* strain Lq80 to weaning piglets stimulates the

growth of indigenous *Lactobacilli* to modify the lactobacillal population. *Journal of General and Applied Microbiology* **53**: 325-332.

Tallon, R., Arias, S., Bressollier, P., and Urdaci, M. C. (2007). Strain- and matrix-dependent adhesion of *Lactobacillus plantarum* is mediated by proteinaceous bacterial compounds. *Journal of Applied Microbiology* **102**: 442-451.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA 4: Molecular evolutionary genetics analysis (MEGA software version 4.0). *Molecular Biology and Evolution* **24**: 1596-1599.

Tannock, G. W., Munro, K., Harmsen, H. J., Welling, G. W., Smart, J., and Gopal, P. K. (2000). Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Applied and Environmental Microbiology* **66**: 2578-2588.

Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Ng, J., Munro, K., and Alatossava, T. (1999). Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. *Applied and Environmental Microbiology* **65**: 4264-4267.

Temmerman, R., Huys, G., and Swings, J. (2004). Identification of lactic acid bacteria: Culture-dependent and culture-independent methods. *Trends in Food Science & Technology* **15**: 348-359.

Temmerman, R., Scheirlinck, I., Huys, G., and Swings, J. (2003). Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* **69**: 220-226.

Tilsala-Timisjärvi, A., and Alatossava, T. (1998). Strain-specific identification of probiotic *Lactobacillus rhamnosus* with randomly amplified polymorphic DNA-derived PCR primers. *Applied and Environmental Microbiology* **64**: 4816-4819.

Timmerman, H. M., Veldman, A., van den Elsen, E., Rombouts, F. M., and Beynen, A. C. (2006). Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. *Poultry Science* **85**: 1383-1388.

Tuomola, E. M., and Salminen, S. J. (1998). Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *International Journal of Food Microbiology* **41**: 45-51.

Vahjen, W., Jadamus, A., and Simon, O. (2002). Influence of a probiotic *Enterococcus faecium* strain on selected bacterial groups in the small intestine of growing turkey poult. *Archives of Animal Nutrition* **56**: 419-429.

Van den Abbeele, P., Grootaert, C., Possemiers, S., Verstraete, W., Verbeken, K., and Van de Wiele, T. (2009). In vitro model to study the modulation of the mucin-adhered bacterial community. *Applied Microbiology and Biotechnology* **83**: 349-359.

van Rijsbergen, C. (1979). *Information Retrieval* 2nd Edition. (London: Butterworths).

Vaughan, E. E., Schut, F., Heilig, H. G., Zoetendal, E. G., de Vos, W. M., and Akkermans, A. D. (2000). A molecular view of the intestinal ecosystem. *Current Issues in Intestinal Microbiology* **1**: 1-12.

Vélez, M. P., De Keersmaecker, S. C., and Vanderleyden, J. (2007). Adherence factors of *Lactobacillus* in the human gastrointestinal tract. *FEMS Microbiology Letters* **276**: 140-148.

Velge, P., Bottreau, E., Quéré, P., Pardon, P., Nicolle, J. C., Morisson, M., Bout, D., and Dimier, I. (2002). Establishment and characterization of partially differentiated chicken enterocyte cell clones. *European Journal of Cell Biology* **81**: 203-212.

Vesterlund, S., Paltta, J., Karp, M., and Ouwehand, A. C. (2005). Measurement of bacterial adhesion - in vitro evaluation of different methods. *Journal of Microbiological Methods* **60**: 225 - 233.

Wagner, R. D. (2006). Efficacy and food safety considerations of poultry competitive exclusion products. *Molecular Nutrition & Food Research* **50**: 1061-1071.

Wang, B., Wei, H., Yuan, J., Li, Q., Li, Y., Li, N., and Li, J. (2008). Identification of a surface protein from *Lactobacillus reuteri* JCM1081 that adheres to porcine gastric mucin and human enterocyte-like HT-29 cells. *Current Microbiology* **57**: 33-38.

Western Cape Department of Agriculture, Institute of Animal Production. (2005). Animal Production: Animal Research in the Western Cape [Online]. Available at: <http://www.elsenburg.com/trd/animalprod/projects/ostrich/braaikuiken.html> [21 March 2010].

Wheeler Alm, E., Oerther, D. B., Larsen, N., Stahl, D. A., and Raskin, L. (1996). The oligonucleotide probe database. *Applied and Environmental Microbiology* **62**: 3557-3559.

Wintzingerode, F. V., Göbel, U. B., and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews* **21**: 213-229.

Wise, M. G., and Siragusa, G. R. (2007). Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *Journal of Applied Microbiology* **102**: 1138-1149.

Yanisch-Perron, C., Viera, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**: 103-119.

Zhou, H., Gong, J., Brisbin, J. T., Sanei, B., Sabour, P., and Sharif, S. (2007). Appropriate chicken sample size for identifying the composition of broiler intestinal microbiota affected by dietary antibiotics, using the polymerase chain reaction-denaturing gradient gel electrophoresis technique. *Poultry Science* **86**: 2541-2549.

Zhu, X. Y., Zhong, T., Pandya, Y., and Joerger, R. D. (2002). 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Applied and Environmental Microbiology* **68**: 124-137.